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Actinomycetes from Mountains of Hadhramout – Yemen

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A B S T R A C T

In this study, the soil samples were collected from four different mountain sites of Hadhramout governorate in Yemen to investigate the diversity of actinomycetes. Soil samples were analyzed for physicochemical characteristics (temperature, pH and nature of the soil samples). Site soil color varied from brown to light brown and dark brown, temperature of the locations ranging from 33ºC to 39.1ºC and pH from 7 to 9. Actinomycetes were isolated by serial dilution and plating method on starch casein agar media. Total 56 species were isolated on the bases of colony characteristics on starch casein agar and all the isolates were screened for antibacterial activity by agar disk diffusion method (ADD) and agar well diffusion method (AWD). So, 30% isolates have shown activity against *Bacillus subtilis* by ADD and 14% isolates by AWD. For *Staphylococcus aureus* 24% isolates had activity by ADD and 8% isolates had activity by AWD. There were 33% isolates showing activity against *Pseudomonas aeruginosa* by ADD and 15% isolates by AWD. The activity was low against *Escherichia coli*, only 24% isolates have shown activity by ADD and 11% isolates have shown activity by AWD. In this study, we noticed MKr123/1 isolate has a strong activity against both Gram positive and Gram negative bacteria so that MKr123/1 isolate was selected and it is identified as *Nocardia otitidiscaviarum*.

Introduction

Hadhramout governorate lies in the eastern part of Yemen. It is comprised of different topography distributed between coastal plains containing beautiful shores on the Arabian Sea, mountains and hills of heights reaching 2000 meters above the sea level. Hot tropical climate in Hadhramout, temperature reaches 40ºC in summer in the interior areas, where continental dry climate prevails, temperature reaches 36ºC in the coastal areas, due to seasonal winds saturated with moisture, the temperature in winter tends to be moderate in the coastal areas, 20-24ºC and 17-20ºC in the interior areas. Actinomycetes are filamentous, Gram positive bacteria that form branching filaments with hyphae and asexual spores. They were originally incorrectly classified as fungi because they possess true aerial hyphae

and form spores, both of which are considered to be fungal characteristics (Lechevalier and Lechevalier, 1967). Actinomycetes are one of the most widely distributed groups of microorganisms in nature, forming a large part of the microbial population of the soil and aquatic environment such as rivers, lakes and other freshwater habitats (Good fellow and Williams, 1983). Actinomycetes are currently known to produce over 10,000 bioactive compounds, 7,600 of which have been isolated from streptomycetes and 2,500 from non-streptomycetes, notably from the so called rare actinomycetes (Lazzarini *et al.*, 2000). Growing problem with screening in several years is the difficulty in rediscovery of already known bioactive compounds (Nolan and Cross, 1988).This shows that the easily accessible microorganisms in soil had been exhausted and must search for microorganisms from unknown sources (Igarashi, 2004). One way to address this problem is to expand the source of actinomycetes from other than terrestrial soils. There is also growing interest in the non-streptomycetes actinomycetes as sources of novel compounds (Nolan and Cross, 1988). The study of actinomycetes strains from mountain soils are important because they are an extreme environment which has not been extensively studied but is believed to have a large diversity of organisms that have not yet been discovered. On this basis, it is presumed that the chances of isolating novel actinomycetes from these soils are high.

Materials and Methods

Soil samples

Ten soil samples were collected from four mountain sites of different regions of Government Hadhramout – Yemen (Fig.1 & 2). The top layer of soil was removed and taken in sterile polyethylene bags. Samples were air dried at room temperature and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bag at room temperature before use. The physical and chemical analyses of the soil samples were carried out by using standard methods (Nawani, 2002) and (Shejul, 1998).

Isolation and culture condition

For each collection sample, 1g of the soil sample was suspended in 10ml and plated on Starch Casein Agar (SCA, pH 7). The inoculated plates were incubated for 2 weeks at 37°C. The suspected colonies for actinomycetes were selectively isolated and transferred to SCA with the help of loop inoculum method. (Kanavade, 2003). Morphology and color of the MKr colony isolate was determined by inoculating on different media and incubated at 28°C for 21 days (Table 1) (Shirling and Gottlieb, 1972). And diffusible pigmentation was determined on glycerol asparagine agar (GAA) and tyrosine agar (TA) for melanin pigment formation. Isolates were inoculated at 28°C for 21 days and observed formation of pigment (Williams and Wellington, 1982a).Morphological characters of the MKr isolate was grown on following media: (1) Starch Casein Agar (SCA) (Nawani, 2002), (2) Glycerol Asparagine Agar (GAA) (Kanavade, 2003), (3) Nutrient Agar (NA) (Goodfelow and O'Donnel, 1989), (4) Yeast extract -Malt extract Agar (YMA), (5) Czapeck's Dox-Thom Agar (CDTA) (Collins *et al.*, 1995) and (6) Sabouraud's Dextrose Agar (SDA) of pH 7.0. The inoculated plates were incubated at 28°C for 7-14 days and observed for colony characters, sporulation and pigmentation (Holt *et al.*, 1994).

Screening of antimicriobial activity

All actinomycetes isolates were screened for their antimicrobial activity by two techniques: agar disc diffusion method (ADD) and agar well diffusion method (AWD). The target bacterial cultures used were *Escherichia coli,*

Staphylococcus aureus, Bacillus subtilis and *Pseudomonas aeruginosa* (Pisano *et al.*, 1992). Based on their antimicrobial properties, isolates were chosen for further biochemical and morphological characterization.

Characterization of actinomycetes

The strong actinomycetes isolates selected from screening were characterized by morphological, biochemical and physiological methods. The morphological method consists of macroscopic and microscopic characterization (Kawato and Sinobu 1979). Various biochemical tests performed were activity of catalase and oxidase, carbon source utilization, nitrate reduction, starch hydrolysis, urea hydrolysis, gelatin hydrolysis and the physiological tests included motility, NaCl resistance, temperature tolerance and pH tolerance.

Results and Discussion

Soil samples collected from mountains had varied pH, temperature and soil color (Table 1). The present study clearly indicated that physico-chemical parameters influenced the biodiversity and distribution of actinobacteria in some mountain Hadhramout Yemen. Same observation was made by a scientist, who found that the distribution of actinobacteria was influenced by physico-chemical parameters such as the soil color, pH and temperature which were noted to influence the growth rate of microorganisms (Al-Mahdi, 2005). From 56 actinomycete isolates, 19 isolates were selected for the study according to color and their activity against bacterial pathogens (Fig.3). All the isolates were screened for antimicrobial activity, by ADD and AWD. Antimicrobial activity was studied against Gram negative and Gram positive bacteria. The target cultures used were *E. coli, P. aeruginos*a. *S. aureus,* and *B. subtilis*. Out of 56 actinomycete isolates, there were 30% isolates showing activity against *B. subtilis* by ADD and 14% isolates by AWD. For *S. aureus* 24% isolates had activity by ADD and 8% isolates had activity by AWD. There were 33% isolates showing activity against *P. aeruginos*a by ADD and 8% isolates by AWD. The activity was low against *Escherichia coli*, only 24% isolates showed activity by ADD and 11% isolates showed activity by AWD as shown in Fig.4. The ADD method used for screening antibacterial was more effective and gave better results than AWD as shown in Fig.5. Similar results were reported by, AL-Mekhlafi (2007), who found the activity in liquid media decreased in comparison with that shown by the method of agar blocks. Frequency of occurrence of actinomycetes with their pH and temperature is shown in table 1. For *B. subtilis* the maximum inhibition zone diameter was 28mm by ADD technique but by AWD the zone was15mm. For *S. aureus* the maximum inhibition zone was 26mm by ADD technique and 12mm by AWD. For *E. coli* the maximum inhibition zone diameter was 24 mm by ADD technique, but was 14 mm by AWD. The inhibition zone *of P. aeruginosa* was 27 mm by ADD, but was 13 mm by AWD (Fig.6).

In this study, we noticed MKr123/1 isolate had strong activity against both Gram-positive and Gram-negative bacteria, so MKr123/1 isolate was selected for other studies (Fig.7). Our isolated strain was identified based on their colony morphology and microscopic morphology (Table 2). The mycelium structure, color, and arrangement of spore were observed by cover slip technique. Similar method has been followed by Berd (1973) and Mansour (2003). In this study, MKr12/1 was taxonomy based on the morphological, physiological and biochemical properties (Tables 3-5 &7) according to Waksman (1953) and Long (1994).

Fig.1 Map of the sample regions

Fig.2 Hadhramout sample sites

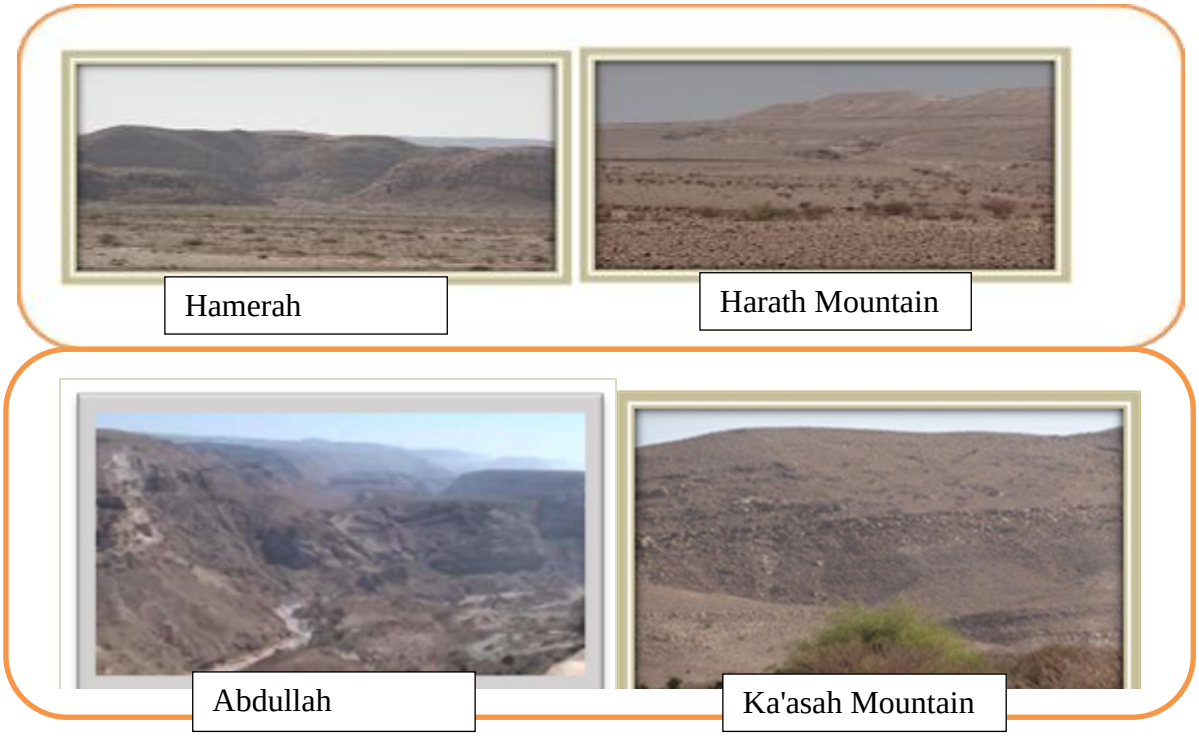

Fig.3 Selection of actionmycetes from different soils

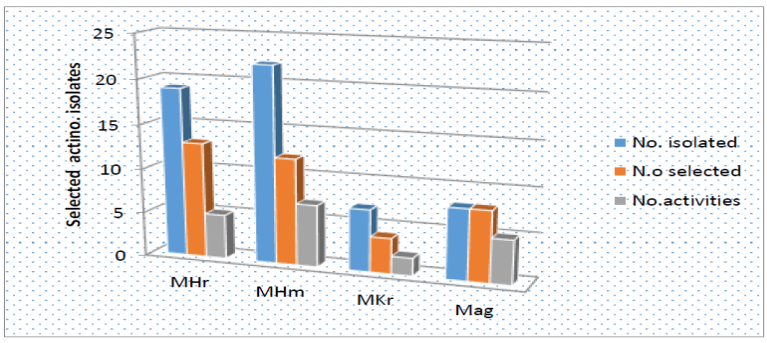

Fig.4 Percentage of antibacterial activity of actinomycete isolates

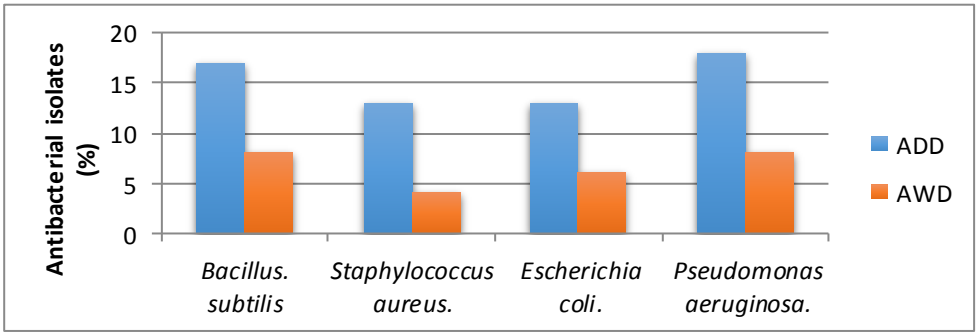

Fig.5 Maximum inhibition zone of antibacterial activity of actinomycete isolates by (ADD) and (AWD)

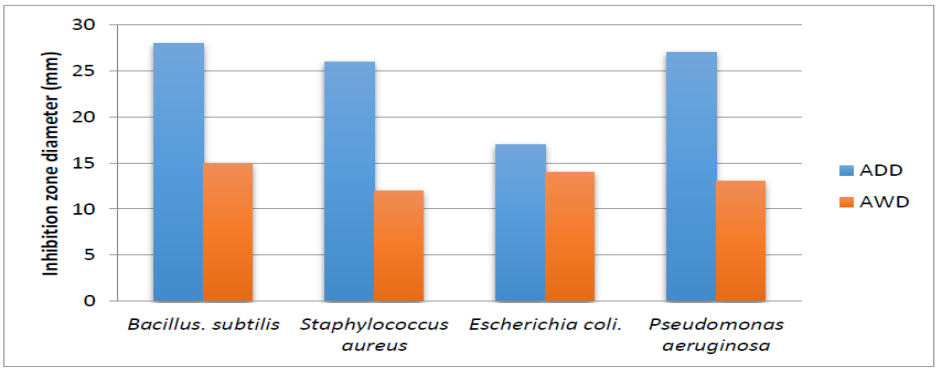

Fig.6 Antibacterial activity by ADD and AWD method

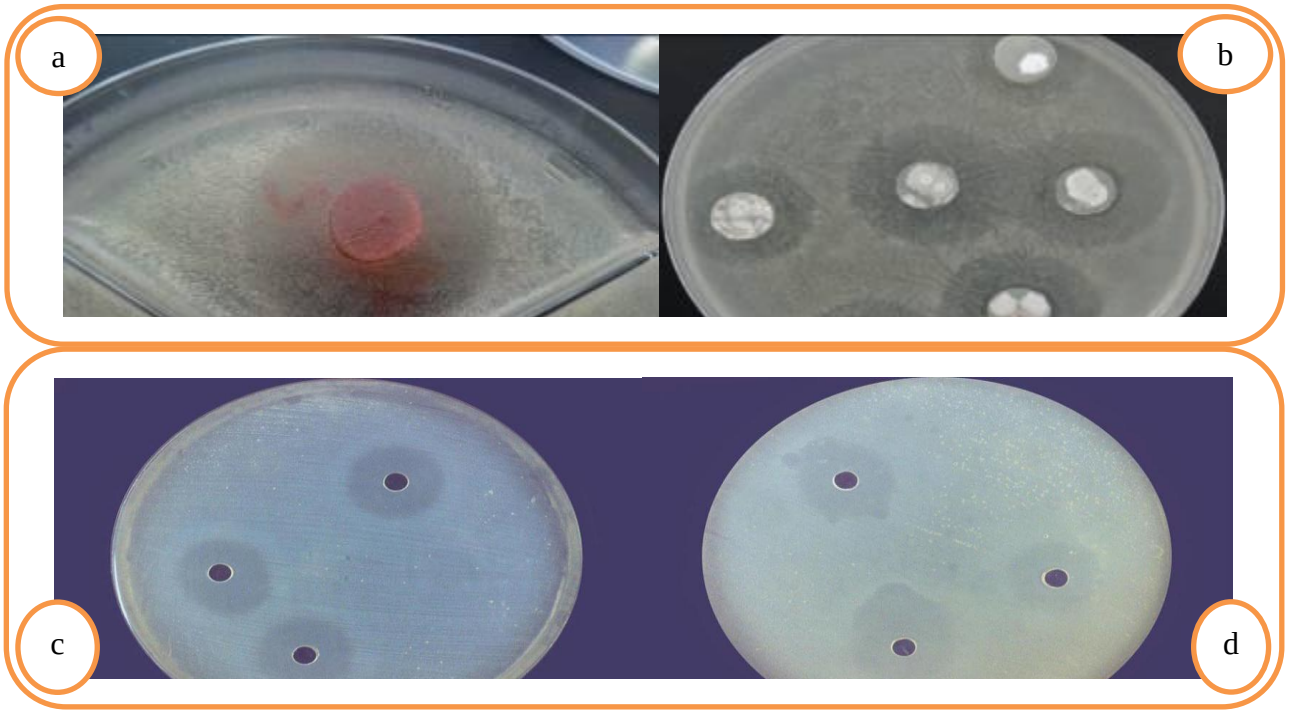

Fig.7 Colony and spore morphology of MKr12/1isolate

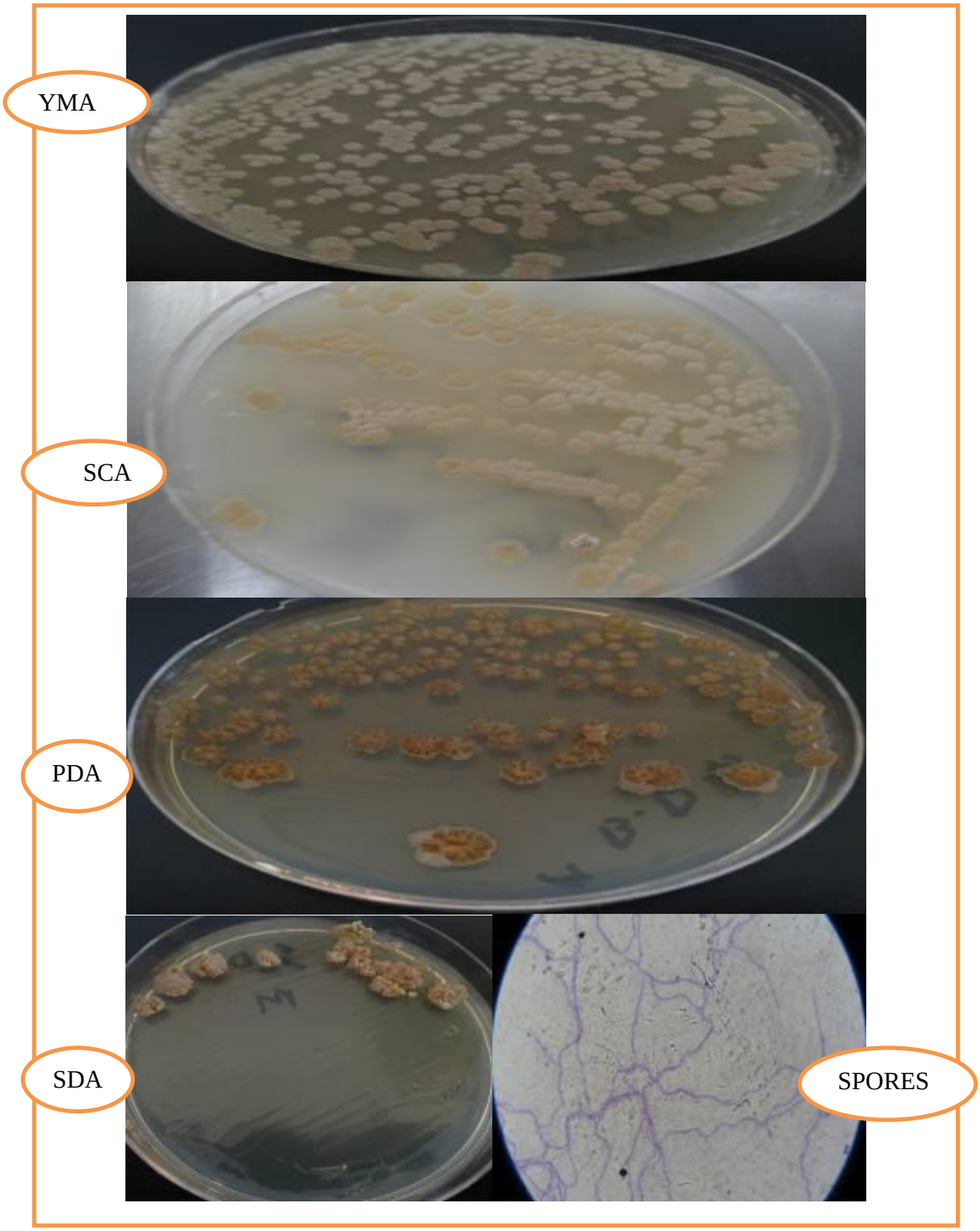

Table.1 Frequency of occurrence of actinomycetes and their pH and temperature

Organism name	Color	Source	Location	pH	Temperature ºC
MHr	Brown	Hadhramout	Harass	9.0	39.1
Mag	Light brown	Hadhramout	RasHawara	7.9	29.0
MHm	Dark brown	Hadhramout	Hamerah	7.9	33.0
MKr	Light brown	Hadhramout	Ka'asah	8.3	33.5

Table.2 Spore and colony morphology of actinomycete isolates

Colony Characters	Colony shape	Colony consistency	Colony elevation	Time of Colony Visible
MKr12/1	Irregular	Hard	Convex	48 hrs.
	Spore Grouping	Spore motility	Spore color	Spore Chain
	Chain	Non motile	Beige	Spiral

Table.3 Growth and cultural characteristics of MKr12/1 on different media

Culture medium	**Growth**	**Arial mycelium**	**Substrate mycelium**	**Soluble pigment**
Starch casein agar	Moderate	None	Beige	None
Nutrient agar	Poor	None	Beige	None
Sabouraud dextrose agar	Poor	None	Saffron yellow	None
Potato dextrose agar	Good	None	Orange brown	Pale brown
Yeast- malt extract agar	Good	None	Maize yellow	None
Glycerol- asparagine agar	Moderate	White yellow	Cream	None
Tyrosine agar	Good	White	Orange brown	Brown
Czapeck's –dox- thom agar	Moderate	None	Cream	None
Glycerol yeast extract agar	Good	None	Beige	None
Starch yeast extract agar	Good	None	Yellow orange	None

Table.4 Physiological characters of MKr12/1isolate

Physiological characters	**MKr12/1**
Temperature:	
20 °C.	+
28 °C.	+
37 °C.	+
45 °C.	-
60 °C.	-
pH:	
3.5	-
4.5	+
7.0	+
10.0	+
NaCl Tolerance:	
0%	+
2%	+
4%	+
6%	+
8%	+
10%	

+ Growth; - No growth

Table.5 Sensitivity of actinomycete isolates to antibiotics (zone of inhibition mm)

Antibiotic	MKr12/1 (mm)
Gentamicin (10 µg)	28
Amphotericin B (100µg)	+
Flucanazole (10 µg)	+
Novobiocin (30µg)	30
Penicillin G (10 µg)	40
Nystatin (10 µg)	+
Clotrimazol (10µg)	38
Co-trimazole (25 µg)	10
Tetracycline (30 µg)	28

Table.6 Results showing utilization of carbon and nitrogen sources

Utilized carbon source	MKr12/1
D- Glucose	+++
Citric acid	±
Lactic acid	±
Cellulose	+++
Lactose	+++
Dextrose	+++
D- Mannose	+++
D- Fructose	++
Sucrose	+++
L- Arabinose	+++
D- Mannitol	+++
Sodium acetate	+
Starch	+++
Glycerol	++
D- Maltose	+++
Basal medium	+
Nitrogen source	
L-Asparagine	+++
L-Tyrosine	+
Glycine	+
Ammonium sulphate	+++
Sodium nitrate	+++
Basal medium	+
H_2S production	**-**
Acid fast reaction	**+**

+++: Very good; ++: Good; +: Poorly; -: Negative; ±: Moderate

Table.7 Enzyme activity of actinomycete isolates

Enzymes	MKr12/1
Amylase	-
Caseinase	-
Catalase	+
Chitinase	+
Gelatinase	-
Keratinase	+
Lecithinase	+
Lipase	-
Pectinase	-
DNase	+
Urase	+

+: Positive test; - : Negative test

So, the MKr was identified as *Nocardia otitidiscaviarum*. Substrate mycelium was colorless and aerial mycelium form short spore chains. The spore mass was yellow to orange. Diffusible pigment was produced. The isolates tolerated 5% NaCl. The isolates utilized chitin, keratin, pectin and cellulose. The isolates utilized mannose, fructose and mannitol as carbon sources (Table 6).

Distribution of actinobacteria was influenced by physico-chemical parameters such as the soil type, soil color, pH and temperature. So, an attempt was made to expand the source of actinomycetes by carrying out ecological assessments of environments other than terrestrial soils. The agar disc diffusion method was more effective for antibacterial activity than agar well diffusion method against bacterial pathogen because liquid medium is fragmented into rods and cell morphology plays an important role in the production of antibiotics. Starch casein medium was the best media for production of antibiotics at pH7.

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