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Isolation and Identification of Bioactive Actinomycete Isolates from Yemen Soils

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ABSTRACT

A total of 50 actinomycete isolates were isolated from different locations in Yemen (Sana'a, Taiz, Ibb and Alhodida). Agar disc diffusion method that used for screening antibacterial and antifungal activities were more effective and give better results than agar well diffusion method. Antibacterial activity of isolates was more effective than antifungal activity. All five selected isolates for study were active against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Microsporium canis*. According to taxonomy of actinomycetes (Morphological, physiological, biochemical and chemotaxonomy characters**)**, all selected isolates were identified as members belonging to genus *Streptomyces* (*S. glaucescenes*, *S. luridus*, *S. antibioticus*, *S. exfoliatus* and *S. filipinensis*).

INTRODUCTION

Actinomycetes constitute a significant proportion of the microbial population in most soils and their viable count often exceeds 1 million per gram (McCarthy and Williams, 1990). Among soil inhabitants, actinomycetes and specifically, *Streptomycetes* are of practical importance because they produce most of the useful natural antibiotics for medical use (Taddei *et al*., 2005). Actinomycetes have the ability to synthesize many different biological active secondary metabolites such as antibiotics, herbicides, pesticides, antiparasitic, immunomodulators and enzymes (Moncheva *et al*., 2002; Oskay *et al*., 2004). More than 50% of the known natural antibiotics produced by actinomycetes. They are a proven source of structurally diverse secondary metabolites possessing broad ranges of biological activities. Example include antibiotic (erythromycin and tetracycline), anticancer (mitomycin and daunomycin), immunosuppressant (rapamycin and FK506) and veterinary agent (thiostrepton and monensin) (Miyadoh, 1993; Moore *et al*., 1999).

MATERIALS AND METHODS

1. Sampling procedure:

Soil samples were collected from different location in Yemen (Sana'a, Taiz, Ibb and Alhodida). The samples were taken from up to 20 cm depth, after removing approximately 10 cm of the soil surface. The samples were placed in sterile plastic container (Aghighi *et al*., 2004).

2. Isolation of actinomycetes:

Isolation of actinomycetes was performed by soil dilution plate technique using starch nitrate agar. Plates were incubated at 28°C for 14 days. The isolates were enumerated and selected for further study (Aghighi *et al*., 2004).

3. Screening of actinomycetes for antimicrobial activity:

-Test microorganisms as bacteria (*Bacillus subtilis* NCTC 10400, *Staphylococcus aureus* isolated from wound and *Escherichia coli* isolated from urine) and fungi (*Candida albicans* MTCC 227 and *Microsporium canis* isolated from hair).

- Antimicrobial activities of actinomycetes performed by agar disc and well diffusion methods. Antagonistic activity was measured by the size of the inhibition zone (Pisano *et al*., 1992; Tepe *et al*., 2004).

4. Identification of bioactive actinomycete isolates:

I. Actinomycete isolates characters:

Five actinomycete isolates were selected according to the maximum bioactive activity and characterized by the following characters:

a. Morphological characters:

- **Cultural characters:** morphology and color of the spores were observed by inoculating the isolates on starch nitrate agar and incubated at 28°C for 21 days (Shirling and Gottlieb, 1972).
- **Spore chain morphological characters:** were determined by cover slip technique according to the categories of Pridham *et al*., 1958 which modified by Shirling and Gottlieb, 1966.
- **Spore surface ornamentation:** was studied by scanning electron microscopic according to (Tresner *et al*., 1961; Dietz and Methews, 1971).
- **Melanin pigment production:** peptone-yeast extract-iron agar and tyrosine agar was used for the detection of deep brown to black melanin diffusible pigment (Shirling and Gottlieb, 1966).

b. Physiological and biochemical properties:

- **Growth at different temperatures:** temperature for the isolates growth was determined on starch nitrate agar media at 25, 30, 37 and 45°C (Kokare *et al*., 2004; Dastager *et al*., 2006).
- **NaCl tolerance of the isolates:** were determined on starch nitrate broth containing sodium chloride ranging from concentration 0-10% (Kokare *et al*., 2004; Oskay *et al*., 2004).
- **Antimicrobial activity:** was determined by agar well diffusion method using two Gram +ve bacteria (*Bacillus subtilis, Staphylococcus aureus*) and one Gram –ve

bacteria (*Escherichia coli*), fungi (*Microsporium canis*) and yeast (*Candida albicans*) as test microorganisms (Tepe *et al.,* 2004).

- **Degradation of different substrates:** hydrolysis of starch according to Collins *et al*., 1995, pectin hydrolysis according to Shejul, 1998, degradation of lecithin according to method used by Kanavade, 2003 and degradation of lipid according to method used by Deshmukh, 1997.
- **Sensitivity to different antibiotics:** actinomycete isolates were tested for antibiotic sensitivity in the presence of antibiotics as neomycin (30 µg), penicillin G (10 units), novobiocin (30 µg), ampicillin (10 µg) by method described by Kokare *et al*., 2004.
- **Nitrogen utilization of the isolated actinomycetes:** was determined by growth on basal medium supplemented with 0.1% nitrogen sources such as L-cystein, DL-phenylalanine, L-tyrosine, L-arginine, L-valine, L-histidine and sodium nitrate (Langham *et al*., 1989).
- **Carbohydrate utilization of the isolates:** was determined by growth on basal medium supplemented with 1% carbon sources such as D (+) sucrose, melibiose, xylose, lactose, D- fructose and rhammnose (Shirling and Gottlieb, 1966).

c. Chemotaxonomic characters: isomers of diaminopimelic acid in the whole cell hydrolysates were determined according to the method Lechevalier and Lechevalier, 1970. Whole cell sugars were analyzed according to the method of Becker *et al*., 1964; Becker, 1965.

II. Identification of actinomycetes: by using Probability Identification of Bacteria (PIB) computer software (Williams *et al*., 1989; Langham *et al.,* 1989).

RESULTS

1. Sampling procedure and isolation of actinomycetes: A total of 50 isolates of actinomycetes were isolated from 6 soil samples (Table 1).

Table (1): Count of actinomycete isolates from different location in Yemen.

Location	Isolate code	No of isolates
Aser-Sana'a, Yemen	A	4
Pit poss- Sana'a, Yemen	B	5
Hada- Sana'a, Yemen	H	8
Alhodida- Yemen	D	6
Taiz-Yemen	T	14
Ibb- Yemen	I	13
Total isolate	-	50

2. Screening of actinomycetes for antimicrobial activity:

Fifty of actinomycete isolates screening for antimicrobial activity by agar disc and well diffusion methods. The agar disc diffusion method was more effectiveness and gives better results than AWD. Out of 50 actinomycete isolates, there were 31 isolates (62%) shown activity against *B. subtilis* by ADD and 21 isolates (42%) by AWD. For *S. aureus* 29 isolates (58%) had activity by ADD and 19 isolates (38%) had activity by AWD. The activity was low against *E. coli*, only 19 isolates (38%) was shown activity by ADD and15

isolates (30%) showed activity by AWD. Only 11 isolates (22%) showed activity against *C. albicans* by ADD and 10 isolates (20%) showed activity by AWD. Against *M. canis*, the activity was very low, only 7 isolates (14%) showed activity by ADD and 4 isolates (8%) showed activity by AWD. The results of the antimicrobial activity of actinomycete isolates are given in table 2.

Table (2): Antimicrobial activity of actinomycete isolates.

Isolate code	Test microorganisms (inhibition zone in cm)									
	B. subtilis		*S. aureus*		*E. coli*		*C. albicans*		*M. canis*	
	ADD	**AWD**	**ADD**	**AWD**	**ADD**	**AWD**	**ADD**	**AWD**	**ADD**	**AWD**
A_1	-	-	-	-	-	-	-	-	-	-
A_2	1.8	-	-	1.7	-	-	-	-	-	-
A_3	-	-	1.9	1.8	-	-	1.7	1.6	-	-
A_4	-	-	1.7	1.6	-	-	-	-	-	-
B_1	-	-	1 .9	1.8	-	-	-	-	-	-
B_2	-	-	-	-	1.9	1.6	-	-	-	-
B_3	2	1 .8	-	-	-	-	-	-	-	-
B_4	1.5	-	1.8	1.6	-	-	-	-	-	-
B_5	-	-	-	-	-	-	-	-	2	-
D_1	1.6	-	-	-	-	-	-	-	-	-
D_2	2.6	-	2 .1	2	-	-	-	-	-	-
D_3	2.5	2.3	1.8	1.6	-	-	-	-	-	-
D_4	2. 4	-	1 .9	1 .7	-	-	-	-	-	-
D_5	2.8	2.5	3	2.5	2.8	2.5	1.9	1.6	1.7	-
D_6	3	2.5	3.3	2.7	1.8	1.6	2.7	2.6	1.9	1.5
H_1	3.4	3	3.5	3	3.5	3.4	2.5	2	2	1.6
H_2	2.1	1.9	1.9	1.6	2	-	-	-	-	-
H_3	-	1.6	1.7	1.6	2.3	1.6	2.1	1.6	-	-
H_4	3.5	2.5	-	-	3.4	2.5	2.1	-	-	-
H_5	-	-	-	-	-	-	1.9	1-.8	-	-
H_6	1.8	-	-	-	-	-	-	-	-	-
H_7	3	2 .5	2 .7	-	-	-	1.8	1.6	-	-
H_8	2.8	2.5	-	-	-	-	1.7	1.6	-	-
I_1	-	-	-	-	-	-	-	-	-	-
I_2	-	-	-	-	-	-	-	-	-	-
I_3	-	-	-	-	-	-	-	-	-	-
I_4	2. 6	1.8	1.6	-	-	-	-	-	-	-
I_5	2	-	2.2	-	-	-	-	-	-	-
I_6	2.6	2.3	2.5	2.4	2.2	2	2.1	1.8	1.8	1.6
I_7	2.8	-	2.2	-	3	-	-	-	-	-
I_8	2	1.6	-	-	-	-	-	-	-	-

I_9	2.4	2	2	-	2.3	-	2	1.6	-	-
I_{10}	2.3	2	1.7	-	-	-	-	-	-	-
I_{11}	3	2	2.4	2	1.9	1.6	1.6	-	-	-
I_{12}	2.1	1.9	2.6	-	2.2	1.8	-	-	-	-
I_{13}	2.2	1.8	2.3	-	2.2	1.7	-	-	-	-
T_1	2.2	1.6	2.5	-	2.5	1.9	-	-	-	-
T_2	1.7	-	-	-	2.8	2	-	-	-	-
T_3	2.2	1.9	3.2	-	2.5	2	-	-	-	-
T_4	-	-	-	-	-	-	-	-	-	-
T_5	-	-	2.6	-	-	-	-	-	-	-
T_6	2.1	1.9	2.2	1.9	2	1.7	1.9	1.6	1.8	1.7
T_7	-	-	-	-	-	-	-	-	-	-
T_8	2	1.5	2.5	1.6	1.5	-	-	-	-	-
T_9	-	-	-	-	-	-	-	-	-	-
T_{10}	-	-	-	-	-	-	-	-	-	-
T_{11}	1.9	-	2	2	-	-	-	-	-	-
T_{12}	-	-	-	-	-	-	-	-	-	-
T_{13}	-	-	1.8	1.6	2.3	2	-	-	1.6	-
T_{14}	-	-	-	-	-	-	-	-	-	-

ADD: Agar Disk Diffusion **AWD**: Agar Well Diffusion

3. Actinomycete isolates characters:

I. Cultural characteristics:

All five isolates grew on agar media showing morphology characters typical as *Streptomyces* genus. Cultural characteristics of isolates on starch nitrate agar are summarized in table (3).

Table (3): Cultural characteristics of five isolates on starch nitrate agar.

Characters / **Isolates**	**Growth**	**Aerial mycelium**	**Substrate mycelium**	**Diffusible pigment**
D_5	Good	White	Violet	Violet
D_6	Good	White to green	Grey	_
H_1	Good	Grey	Brown	_
I_6	Good	White	Brown	Dark brown
T_{10}	Good	White to pink	Orange	Orange

II. Morphological, physiological and biochemical characteristics:

The morphological, physiological and biochemical characters of five bioactive isolates are summarized in table (4).

III. Chemotaxonomic analysis:

Analysis of the whole-cell hydrolysate of five isolates showed the presence of a cell wall chemotype I by presenting of L-DAP and no diagnostic sugars were found.

Table (4): Morphological, physiological, biochemical and chemotaxonomy characters of five isolates.

Characteristics	Isolates				
	D_5	D_6	H_1	I_6	T_{10}
Aerial mycelium	+	+	+	+	+
Spore chain character:					
Spirals	+	+	-	-	+
Hook	-	-	-	+	-
Flexuous	-	-	+	-	-
Spore surface ornamentation:					
Wavy	+	-	+	ND	-
Hairy	-	+	-	ND	-
Spiny	-	-	-	ND	+
Spore mass color:					
White	+	-	-	+	+
Grey	-	+	+	-	-
Mycelium pigment red-orange	+	-	-	-	+
Diffusible pigment produced	+	-	+	+	+
Diffusible pigment yellow-brown	-	-	+	+	-
Melanin production on media:					
Peptone yeast extract iron agar	-	+	-	+	+
Tyrosine agar	-	-	-	-	-
Growth at:					
Temperature:					
25°C	+	+	+	+	+
30°C	+	+	+	+	+
37°C	+	+	+	+	+
45°C	+	+	+	-	-
NaCl:					
NaCl 6%	+	+	+	+	-
NaCl 8%	+	-	+	-	-
Antagonistic activity:					
B. subtilis	+	+	+	+	+
S. aureus	+	+	+	+	+
E. coli	+	+	+	+	+
C. albicans	+	+	+	+	+
M. canis	+	+	+	-	+
Degradation of:					
Lecithin	+	+	+	+	+
Lipid	+	+	+	+	+
Pectin	+	+	+	+	+

Xylan	+	-	+	+	+
Urea	+	+	-	-	-
Resistance to:					
Neomycin (30 µg)	-	-	-	-	+
Penicillin G (10 units)	+	+	+	+	+
Novobiocin (30 µg)	-	-	-	-	+
Ampicillin (10 µg)	+	-	+	+	+
Utilization of:					
L-cystein	+	+	-	-	+
DL-phenyl alanine	+	+	-	+	+
L-tyrosine	+	+	+	+	+
L-arginine	+	+	+	+	+
L-valine	+	+	+	+	+
L-histidine	+	+	+	+	+
Sodium nitrate	+	+	+	+	+
D(+)sucrose	+	+	+	+	+
Melibiose	+	+	+	+	+
Xylose	+	+	+	+	+
Lactose	+	+	+	+	+
D-fructose	+	+	+	+	+
Rhammnose	-	+	-	+	-
Chemotaxonomy:					
DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP
Diagnostic sugar	-	-	-	-	-

+, positive; -, negative; ND, not determined.

4: Identification of actinomycetes by PIB software: Table 5 show the identification of actinomycete isolates by PIB software.

Table (5): Identification of actinomycete isolates by PIB software.

Isolate code	Name
D_5	*Streptomyces glaucescenes*
D_6	*Streptomyces luridus*
H_1	*Streptomyces antibioticus*
I_6	*Streptomyces exfoliatus*
T_{10}	*Streptomyces filipinensis*

DISCUSSION

From 6 soil samples collected from different sites in Yemen, 50 isolates of actinomycetes were found. Actinomycete isolates were screened for antimicrobial activity by two methods, viz., ADD and AWD. All isolates showed antimicrobial activity on ADD but failed to do so in fermentation media. This was correlated with the morphology of the culture. It could maintain filamentous form on the agar medium, while in the liquid medium it fragmented into rods. This has suggested that the cell morphology plays an important role in the production of antibiotics which recorded by Shomura *et al.* 1979. According to antimicrobial activity and spectrum broadness, 5 isolates were selected and identified

depend upon maximum antimicrobial activities. Morphological examination of the 5 isolates clearly indicates that these isolates belong to the *Streptomyces* genus (Waksman, 1961; Cross, 1989; Goodfellow, 1989; Lechevalier, 1989; Locci, 1989; Williams *et al*., 1989). Further comparison of physiological and biochemical characteristics of the isolates after used software PIB for identification indicated that the D_5 is similar character to *Streptomyces glaucescenes* (98%). Isolate D_6 isolate as *Streptomyces luridus* (98%). In the same manner, H_1 isolate was identified as *Streptomyces antibioticus* (98%), I_6 isolate as *Streptomyces exfoliatus* (98%) and T_{10} isolate as *Streptomyces filipinensis* (98%).

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عزل وتعريف الأكتينوميسيتس ذات النشاط الضد ميكروبى من التربه اليمنية

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ملخص

تم فى هذه الدراسة جمع 50 عزله من الاكتينوميسيتس عزلت من مواقع مختلفة من اليمن (صنعاء, تعز, اب و الحديدة). وقد أظهرت طريقة انتشار أقراص الأجار التي استخدمت لدراسة النشاط الضد البكتيري و الضد الفطري أكثر تأثيرا و تعطي نتائج أفضل من طريقة انتشار حفر الأجار. كما أن نشاط العزلات ضد البكتيريا أكثر فاعلية من نشاط العزلات ضد الفطريات. كل العزلات المختارة للدراسة لها نشاط ضد *Bacillus subtilis*, *Staphylococcus aureus* , *Escherichia coli* , *Candida albicans* و *Microsporium canis* .

تبعا لتصنيف الاكتينوميسيتس (الصفات المورفولوجية, الفسيولوجية, الكيمائية الحيوية و التصنيف الكيميائي), كل العزلات عرفت كأعضاء تابعة لجنس *Streptomyces* (*S. glaucescenes* , *S. antibioticus*, *S. luridus* , *S. exfoliatus* و *S. filipinensis*).