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Characterization of Actinomycetes from Soil Sample of Nahargarh Hill Area, Jaipur, India for Production of Antimicrobial Compounds and Enzymes

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ABSTRACT:

Actinomycetes play an important role in the decomposition of various organic materials and replenish the supply of nutrients in the soil. They also produce many secondary metabolites possessing antimicrobial properties. The present study was conducted to determine the antibacterial and enzyme producing capabilities of soil Actinomycetes isolated from Nahargarh hill area, Jaipur, Rajasthan, India. A total of 26 isolates of pigmented Actinomycetes were recovered from soil sample and screened for their antibacterial activity against five bacterial strains. Two of the total isolates possessed broad spectrum activity against all test bacteria. 46% of the isolates showed antagonistic activity against at least one of the test bacterium. Six distinctly pigmented isolates were selected for genus identification studies based on cultural, morphological and biochemical analysis. Results indicated that they belonged to the genus Streptomyces. Preliminary screening for production of hydrolytic enzymes by selected isolates showed that amylase was produced by all isolates. Thus soil Actinomycetes are the potential source of industrially important enzymes and antimicrobial compounds.

Key words: Soil Actinomycetes, antibacterial activity, pigmentation, hydrolytic enzymes, Streptomyces

[I] INTRODUCTION

Actinomycetes are a ubiquitous group of filamentous bacteria with a high GC content in their DNA. They are widely found in natural ecosystems and are of immense importance as they breakdown organic matter and thereby provide nutrients in the soil [1]. In this process they need plenty of secondary metabolites and enzymes that are formed during their own metabolism. From the traditional times they are known to be producers of antimicrobial compounds. Among the various genera of Actinomycetes identified so far, *Streptomyces*

are quite important as secondary metabolite producers (pigments and antibiotics) and hence they are of high pharmacological and industrial interest [2-3]. About two thirds of known naturally produced clinically antibiotics are derived from *Streptomyces* [4]. Antibiotic resistance is one of the most serious public health problems which require immediate attention and thus calls upon the need of finding novel organisms that possess better antimicrobial capacity. The diverse metabolic capabilities enable them to produce a number of enzymes which can be used for different biotechnological applications. They are also known to bring about the decomposition of various polymers (polysaccharides, proteins and lipids) by the production of hydrolytic enzymes and thus are also receiving worldwide attention due to their applications, especially in the biodegradation of agronomic wastes, and are being gradually more used in textile, paper-pulp and food industries [5-8]. The conventional technique of 'random screening' in which the microorganisms are isolated, grown and their activity spectrum is assessed is still expected to produce potential results and thus this traditional approach may prove beneficial [9]. The important approaches helpful in discovering novel Actinomycetes species or unknown bioactive substances (mainly pigments, antibiotics and enzymes) include isolation and characterization of Actinomycetes from the extreme habitats [10] and relatively unknown or unexplored areas [11]. In this regard, **Nahargarh hills** (600 ft), **Amber** (15 km from Jaipur), region of Aravali hills in Rajasthan is of significant interest. Its high altitude and seasonal rainfall create extreme habitation which is likely to harbour unusual types of pigmented Actinomycetes. Since poorly studied habitations increase chances of finding novel Actinomycetes, therefore, present study was undertaken to isolate and characterize pigmented Actinomycetes from soil samples collected from Nahargarh, Jaipur (Rajasthan). These isolates were then screened for their antagonistic properties and enzyme producing abilities.

[II] MATERIALS AND METHOD

2.1. Collection of soil sample

Soil sample was collected from Nahargarh hill area (26°56'4"N; 75°49'3"E) of Aravali mountain ranges, 15 Km from Jaipur, Rajasthan. Its high altitude and seasonal rainfall provides habitat to a very rich ecosystem including diverse variety of microorganisms. The soil samples were aseptically collected, in sterilized petri dishes, stored in iceboxes and transported to the laboratory where they were kept in refrigerator at 4^0C until analysis [12].

2.2. Isolation of Actinomycetes from Soil

Isolation of Actinomycetes was carried out by serial dilution method on glycerol-yeast extract agar (glycerol 5 g/l, yeast extract 2 g/l, K_2HPO_4 0.1 g/l, peptone 25.0 g/l, agar 15 g/l) and Actinomycetes isolation agar (AIA) medium (sodium caseinate 2.0 g/l, L-asparagine 0.1 g/l, sodium propionate 4.0 g/l, Dipotassium phosphate 0.5 g/l, magnesium sulphate 0.1 g/l, ferrous sulphate 0.001 g/l, agar 15 g/l supplemented with 5ml/l glycerol) Both the media were supplemented with Nystatin and Cyclohexamide (50 µg/ml) as antifungal agents [13]. One gram of dried soil was taken in 9 ml of distilled water and agitated vigorously. Different aqueous dilutions, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} of the suspension were spread onto plates containing medium. After gently rotating, the plates were incubated at 30^0C for 10 to 35 days for appearance of Actinomycetes colonies [14]. Dry, powdery and typically pigmented colonies were selected from mixed culture and isolates were purified by using quadrant streak technique and were maintained on fresh AIA medium at 4^0C for further study. The isolates were categorized as slow, moderate and fast growers on the basis of their incubation time.

2.3. Preliminary Screening for antibacterial activity

All the isolated Actinomycetes were screened for antibacterial activity on Nutrient Agar

medium using cross streak method [15] against five bacterial strains as *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 424) and *Proteus vulgaris* (MTCC 426) that were procured from MTCC (Microbial Type Culture Collection), Chandigarh. The isolates were streaked as a straight line in the centre of the petri plate and were incubated at 30^{o}C for 15-25 days. After incubation, the test bacterial strains were streaked at right angle to the original streak of the Actinomycetes isolates. The area of inhibition adjacent to the line of growth against bacterial strains was observed after 24 h of incubation.

2.4. Genus identification of six distinctly pigmented isolates

Six isolates were selected on the basis of their distinct pigmentation and rapid growth. These selected isolates were identified by studying morphological and biochemical characteristics as per the International *Streptomyces* Project (ISP) according to Shirling and Gottlieb [16]. The morphological method consists of macroscopic and microscopic characterization**.** Microscopically, the characteristics were studied by cover slip culture method [17] after 4-6 days of incubation on AIA medium. Colonies were observed for mycelium structure, their fragmentation and spore chain morphology under 100X oil immersion. Gram's staining and Acid Fast Staining [18] was also performed for the isolates. Macroscopic Characters like Colour of aerial and substrate mycelium along with pigment production were observed on different ISP media- ISP 2, ISP 3, ISP 4, ISP 5, and ISP 6 [16]. These observations were compared with Bergey's Manual of Determinative Bacteriology, ninth edition [19]. Various biochemical tests were performed such as Tyrosine utilization test, Xanthine utilization test, Hypoxanthine utilization test, Gelatine liquefaction test, H_2S production test, Carbohydrate fermentation, Indole Production test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test [20].

2.5. Enzymatic screening of six selected isolates

Six selected isolates were screened qualitatively for the production of four important enzymes such as cellulase, amylase, protease, and xylanase by agar plate assay in which the isolates were inoculated on respective media and incubated for 4-6 days at 30^{0}C. Starch agar medium was used for amylase production. After incubation the plates were flooded with iodine reagent [21]. SMP agar medium was used for protease production [22]. CarboxyMethyl Cellulose (CMC) agar medium was used for secretion of cellulase after which the plates were flooded with an aqueous solution of Congo red (1% w/v) for 15 min and then destained with 1.0 M NaCl for 15 min [23]. Xylan agar medium was used for xylanase screening. After incubation, the plates were stained with Congo red solution (0.1 % Congo red and 5% ethanol in distilled water) for 15 minutes and destained with 1M NaCl [24]. The development of clear zone of hydrolysis around isolate was observed after each screening assay as an indicator of hydrolytic activity of isolates.

[III] RESULTS

3.1. Actinomycetes isolation

Twenty six Actinomycetes colonies were isolated after serial dilution method. The selection of Actinomycetes colonies was aided by their unique morphological features as glabrous or chalky, heaped, folded and with aerial and substrate mycelia of different colours.

Isolates were prefixed with "NRGH", an abbreviation of Nahargarh.

3.2. Growth characteristics of isolated Actinomycetes

Actinomycetes are slow growers as compared to other bacteria. All the isolates of soil Actinomycetes were grown on AIA at 30^{o}C and the growth rate was monitored every day up to

35 days. The isolates were categorized on the basis of their incubation time **[Figure-1]**.

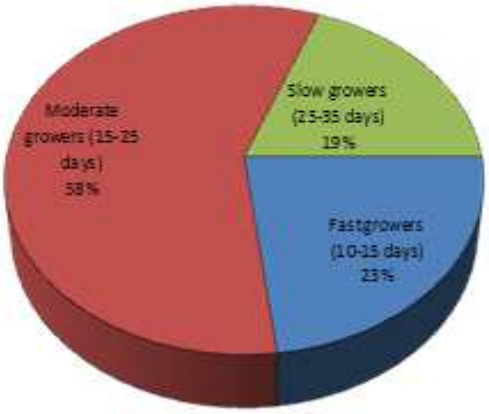

Fig: 1. Growth characteristics of isolated actinomycetes.

3.3. Antibacterial Screening

The screening results for antibacterial activity of all the twenty six Actinomycetes isolates varied **[Table-1]**. Among the isolates tested "NRGH 13" **[Figure-2]** and "NRGH 17" **[Figure-3]** had showed broad spectrum activity. Growth of *Bacillus subtilis* was inhibited by most of the isolates. Growth of *Pseudomonas aeruginosa* was not inhibited by most of the isolates except "NRGH 13". Total number of isolates which showed positive result in antibacterial activity (at least against one test bacteria) was 46.1% **[Figure-4].**

Isolated Actinomycetes	**Test Bacterial Strains**				
	E. coli (MTCC 739)	*S.aureus* (MTCC 737)	*B. subtilis* (MTCC 441)	*P. vulgaris* (MTCC 426)	*P.aeruginosa* (MTCC 424)
NRGH 1	_	_	_	_	_
NRGH 2	_	_	_	_	_
NRGH 3	_	+	+	_	_
NRGH 4	_	_	_	_	_
NRGH 5	_	_	+	_	_
NRGH 6	_	_	_	_	_
NRGH 7	_	_	_	_	_
NRGH 8	_	_	_	_	_
NRGH 9	_	_	+	_	_
NRGH 10	_	_	_	_	_
NRGH 11	_	+	+	_	_
NRGH 12	_	_	_	_	_
NRGH 13	+	+	+	+	+
NRGH 14	_	+	+	_	_
NRGH 15	_	+	+	_	_
NRGH 16	_	_	_	_	_
NRGH 17	+	+	+	+	_
NRGH 18	_	_	_	_	_
NRGH 19	ND	ND	ND	ND	N
NRGH 20	_	_	_	_	_
NRGH 21	_	_	_	_	_
NRGH 22	_	_	+	+	_
NRGH 23	_	_	+	_	_
NRGH 24	_	+	+	_	_
NRGH 30	_	_	+	_	_

(+) = Effective against test bacteria; (-) = Not effective against test bacteria; (ND) = Not Determined

Table: 1. Sensitivity against bacterial strains: results of the isolates screened against five bacterial strains.

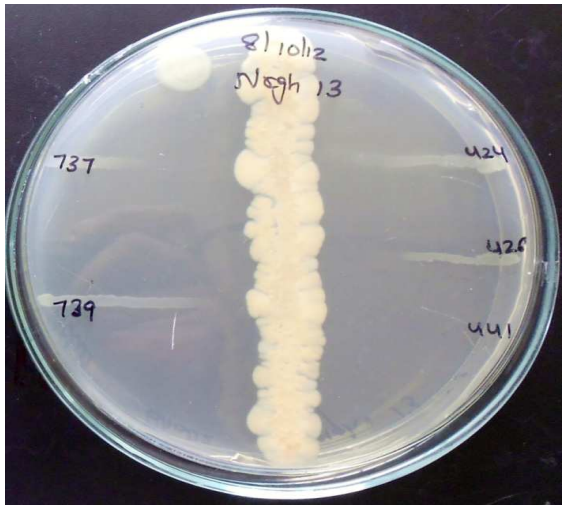

Fig. 2: Antagonistic activity of NRGH 13 against five bacterial strains.

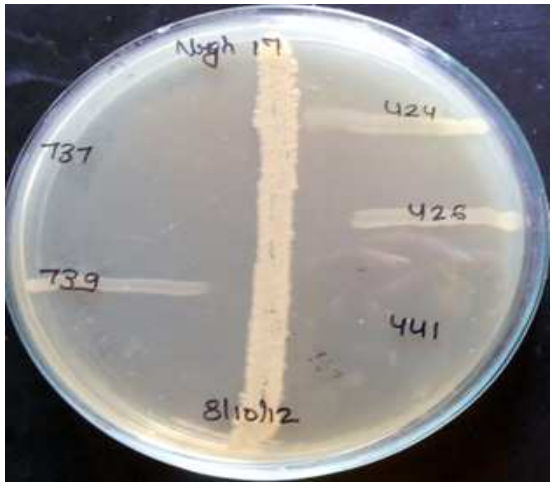

Fig. 3: Antagonistic activity of NRGH 17 against three bacterial strains.

Fig. 4: Antagonistic activity of NRGH 11 against two bacterial strains.

3.4. Macro Morphology of isolated Actinomycetes on AIA medium

All the isolated Actinomycetes colonies were examined for the presence of colour of aerial mycelium and substrate mycelium along with pigment production on AIA medium **[Table-2]**.

Actinomycetes isolates	Color of areial mycelium	Color of substrate mycelium	Diffusible pigment production
NRGH 1	light brown (chalky)	Very light Brown	-
NRGH 3	Dark red	Dark brown	Yellow color
NRGH 4	Light yellow	Cream	Wine color
NRGH 5	Thick white Powder	Purple	Purple color
NRGH 6	Creamy chalk	Light yellow	-
NRGH 7	Light brown (smooth)	Light brown	Wine color
NRGH 8	White	Light brown	-
NRGH 9	Very thick white chalk	Light brown	-
NRGH 10	Olive green Compact	Soil brown	-
NRGH 11	Light yellow	Lemon yellow	-
NRGH 12	White chalk	Cream	-
NRGH 13	Off white	White	-
NRGH 14	Thin white Chalk	Cream	-
NRGH 15	Chalky white	Blood red	Red color
NRGH 16	Red (compact)	Dark brown	-
NRGH 17	Cream	Cream	-
NRGH 18	Light brown	Brown	Wine colour
NRGH 19	Cream	Cream	-
NRGH 20	Foamy white	Blood red	-
NRGH 21	Cream	Light brown	-
NRGH 22	Smoky grey	Dark green	-
NRGH 23	Light grey	Dark green	-
NRGH 24	White	Cream	-
NRGH 25	Light orange	Cream	-
NRGH 30	Chalky White	Light brown	-

Table: 2. Macro morphology on AIA medium

3.5. Genus identification of six selected isolates

Microscopic observations

The colonies of six selected isolates were observed using coverslip culture technique. The colonies of "NRGH 2" were composed of thick network of spiral filaments which break into coccoidal shaped cells. Mycelia of "NRGH 3" were composed of long straight filaments which were found to be the chains of rod shaped cells on 100X oil immersion. Isolate "NRGH 5" was observed to be composed of long spiral filaments which reproduced into chain of cocci at the tip of the filaments. Colonies of Isolate "NRGH 10" appeared as network of flexible filaments surrounded by coccoidal shaped cells. Filamentous growth was also observed in the mycelia of "NRGH 11" and filaments were straight and branched chains. Colonies of Isolate "NRGH 22" were filamentous and short spiral filaments break into coccoids. All the isolates were found to be Gram positive and Acid Fast negative. Results indicated that they belong to genus *Streptomyces*.

Macroscopic characterization

Six pigmented isolates that grew on ISP media (ISP 2 to ISP 6) were slow growing, aerobic, with chalky and folded colonies and aerial and substrate mycelia of different colours **[Table-3]**. Colours of aerial mycelia were observed in the series of red, grey, white, yellow and green on AIA medium but varies on different ISP media. Similarly the substrate mycelium all the isolates showed reverse side pigmentation of

dark brown, red, cream or purple colour. Except NRGH 11, all the isolates showed diffusible pigment production (dark brown colour) on ISP 6 medium thus confirming the presence of melanin production in the medium. Five of the colonies possessed an earthy odour excluding NRGH11.

Characteristics	Medium	NRGH 2	NRGH 3	NRGH 5
Colour of Aerial Mycelium	ISP 2	Chocolate brown (chalky)	No growth	Powdery Peach
	ISP 3	White chalk	Light brown	Peach powder
	ISP 4	Powdery Light grey	Dark brown	Light pink (chalky)
	ISP 5	chalky Peach	Greyish white	Peach (powdery)
	ISP 6	Cream	Light brown	Grey
Colour of Substrate Mycelium	ISP 2	Dark brown	No growth	Blood red
	ISP 3	Dark brown	Dark brown	Dark purple
	ISP 4	Very light brown	Brown	Light red
	ISP 5	Cream	Dark brown	Cream
	ISP 6	light brown	Dark brown	Cream
	ISP 2	-	-	-
	ISP 3	-	Light brown	-
	ISP 4	-	Light Yellow	-
	ISP 5	-	Light yellow	-
	ISP 6	Dark brown	Dark brown	Dark brown

Table: 3(a). Cultural characteristics of three selected isolates on various ISP media

Characteristics	Medium	NRGH 10	NRGH 11	NRGH 22
Colour of Aerial Mycelium	ISP 2	Light grey	No growth	Smoky grey
	ISP 3	White and grey powdery patches	Yellow	Smoky grey
	ISP 4	Grey and white patches (chalky)	Cream	Smoky grey
	ISP 5	Light grey	Light yellow	Grey
	ISP 6	Dark brown	Light yellow	Light brown
Colour of Substrate Mycelium	ISP 2	Dark brown	No growth	Dark brown
	ISP 3	Dark brown	Cream	Dark brown
	ISP 4	Cream	Cream	Light grey
	ISP 5	Off white	Cream	Dark brown
	ISP 6	Grey	Yellow	Brown
Diffusible pigment production	ISP 2	-	-	-
	ISP 3	-	-	-
	ISP 4	-	-	-
	ISP 5	-	-	-
	ISP 6	Dark brown	-	Dark brown

Table: 3(b). Cultural characteristics of three selected isolates on various ISP media

Biochemical characterization of six selected isolates **[Table-4]** showed that some of them were found to degrade tyrosine, xanthine, hypoxanthine, citrate and gelatine also. Only two of the isolates were found to liberate H_2S gas. None of the isolates showed positive result for indole production and methyl red test. As per the utilization of the several carbohydrates, it was noted that all the isolates were able to use dextrose and lactose as sole carbon sources but only few utilized sucrose.

Biochemical test	NRGH 2	NRGH 3	NRGH 5
Tyrosine utilization test	+	_	+
Xanthine degradation test	+	_	+
Hypoxanthine degradation test	+	+	+
Gelatin liquefaction test	+	+	+
H_2S production Test	_	_	+
Carbohydrate fermentation			
a) Lactose	+	+	+
b) Dextrose	+	+	+
c) Sucrose	+	_	_
Indole Production test	_	_	_
Methyl Red test	_	_	_
Voges Proskauer test	+	+	+
Citrate utilization test	_	_	+
Catalase test	+	_	+

(+) Positive result; (-) Negative result

Table: 4(a). Biochemical characters of selected Actinomycetes

Biochemical test	NRGH 10	NRGH 11	NRGH 22
Tyrosine utilization test	_	_	+
Xanthine degradation test	+	_	+
Hypoxanthine	+	_	+

degradation test			
Gelatin liquefaction test	+	_	+
H_2S production Test	_	_	+
Carbohydrate fermentation			
a) Lactose	+	+	+
b) Dextrose	+	+	+
c) Sucrose	_	_	+
Indole Production test	_	_	_
Methyl Red test	_	_	_
Voges Proskauer test	+	+	+
Citrate utilization test	_	_	_
Catalase test	+	_	+

(+) Positive result; (-) Negative result

Table: 4(b). Biochemical characters of selected Actinomycetes

Results obtained after complete examination of Morphological and biochemical observations for all six distinctly pigmented isolates indicated towards genus *Streptomyces* according to the Bergey's Manual of Bacteriology, ninth edition [19].

3.6. Screening of Actinomycetes for production of hydrolytic enzymes

The screening results for enzyme production by selected isolates showed that Amylase was produced by 36%, Cellulase by 29%, protease by 14% and xylanase by 21% **[Table-5]**. These results are helpful in determining the production of industrially important hydrolytic enzymes by the soil Actinomycetes which could be harvested on large scale for commercial use.

Isolates / Enzymes	NRGH 2	NRGH 3	NRGH 5	NRGH 10	NRGH 11	NRGH 22
Amylase	+	+	+	+	_	+
Protease	_	_	+	_	+	_
cellulase	+	-	+	+	_	+
Xylanase	+	_	+	_	_	+

Table: 5. enzymatic activity of selected Actinomycetes isolates.

[IV] DISCUSSION

The screening of natural products from microorganisms continues to indicate a major route to the discovery of new therapeutic compounds and enzymes. Actinomycetes are known to produce many bioactive products in the form of secondary metabolites (antibiotics and pigments) and enzymes of high commercial value and are continue to be routinely screened for new bioactive substances. In the course of isolation of Actinomycetes from soil samples of Nahargarh area, total 26 isolates were obtained and screened for the production of antimicrobial substances. Studied isolates were categorized on the basis of the colour of aerial mycelium as white series (57.6%), brown series (11.5%), grey series (7.6%), red series (7.6%), yellow series (7.6%), green series (3.8%) and orange series (3.8%). The white series isolates were more predominant (57.6% of the total isolates). Preliminary screening for Antibacterial activity of twenty six isolates revealed that broad spectrum of activity was observed in only two (26.6%) of the isolates that were the members of white series. These two can be further studied by secondary screening procedure for the production of antimicrobial compound by fermentation. Moreover it was also observed that most of the isolates showed activity against Gram positive bacteria. Six of the selected isolates (distinctly pigmented) were found to members of the genus *Streptomyces*. Some of the isolates also produced diffusible melanin pigment in the on ISP 6 medium. Actinomycetes are important not only to the pharmaceutical industries but also to the agriculture. They interact in different ways with the higher plants and have potential to utilize different plant polysaccharides such as cellulose, pectin and xylan as carbon sources as they are capable of producing the hydrolytic enzymes [25].

In the present study six selected isolates were screened for their extracellular enzymatic activity of industrially important enzymes such as cellulase, xylanase, protease and amylase on their respective substrate media. Observations revealed that the activity of amylase and cellulase enzymes was possessed by most of the isolates. NRGH 5 was found to produce all the enzymes tested and hence it can be further studied for enzyme quantitation studies. Secondary screening of these isolates for

antimicrobial compound and enzyme production is in process and it is hoped that in the near future we might have some useful products from these isolates of Nahargargh, Jaipur, India.

[V] CONCLUSION

Soil Actinomycetes of Nahargarh hill area in Jaipur, Rajasthan are metabolically active which leads to the production of antibacterial substances and various enzymes. These results indicated that the soils of Nahargarh hill area are a potential source for a wide spectrum of antimicrobial and industrial enzyme producing Actinomycetes. Moreover, it can be an imperative resource for bioprospecting novel/rare *Streptomyces* spp., which could yield valuable bioactive molecules.

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