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Isolation and Screening of Antimicrobial and Extracellular Pigment Producing Actinomycetes from Chambal Territory of Madhya Pradesh Region, India

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Abstract

In present study the objective is to isolate, characterize and study of biological activity of pigment producing actinomycetes. Samples were collected from rhizosphere soil of Chambal territory and other parts of Madhya Pradesh regions. Screening of actinomycetes was done on the basis of pigment diffusion ability in different media. Characterization of the actinomycete was made by scanning electron microscopy and 16s rRNA molecular sequencing. The antimicrobial activity of selected actinomycete was done by over lay agar method and well agar diffusion method against various pathogenic microorganisms.

Among 85 actinomycetes isolates, only ARITM02 showed pigment producing and diffusion ability in media. The Gram staining and scanning electron microscopy confirmed the linear chain structure of actinomycete. Morphological, biochemical and molecular analysis confirmed the isolate belong to genus *Streptomyces*. *Streptomyces* isolate also shown notable antimicrobial activities against various pathogens. These significant results make *Streptomyces* suitable for further investigation and industrial exploitation. Present investigation reveals that Chambal territory region of Madhya Pradesh has great ability to produce potent actinomycetes which possess pigment production and antimicrobial activities against various pathogens.

1. Introduction

Actinomycetes are Gram-positive bacteria and they also known as a good source of microbial secondary metabolites producer in various pharma industries for therapeutic use. It is reported that many synthetic colors cause many serious problems related to environmental and it creates interest towards natural pigments from microorganisms. Natural pigments from actinomycetes are potentially good alternative of synthetic colors. Industrially many artificial synthetic colorants been used in foodstuff, dyestuff, cosmetic and pharmaceutical processes, which have many hazardous effects on health and ecosystems. Due to negative effect of synthetic colorants, there is worldwide interest generated for the production of pigments from natural sources such as microorganisms [1, 2]. Pigments were primarily used as a coloring agent in various industries, researcher have focused the usage of pigments from coloring agents to antioxidants in various pharmaceutical and food industries from the past decade [3]. Some actinomycetes are capable of producing colored substances in the culture media [4]. Actinomycetes are known to be produced many kinds of antibiotics and these antibiotics include many pigments [5]**.** In today's scenario colours have been widely applied in many areas like foods, cloths, paintings, cosmetics, pharmaceuticals and plastics and they also play an important role in food colorant. Pigments have an interesting role in industry for its nanotechnology as it used in bio-plastics and biopolymers [6]. Microbial screening is an important aspect as there is an important source for the production of secondary metabolites that possess clinical and pharmaceutically relevant biological activities. About 22500 biologically active compounds reported till date from microorganisms in which 17% isolated from bacteria, 38% isolated from fungi and 45% isolated from actinomycetes [1]. Actinomycetes are reported as an ecological diverse group. India has wide ecological diversity which constitutes the large microbial population like actinomycetes in soil which producing active secondary metabolites and also the natural pigment producing ability. This geographic diversity of Indian soil offers the variability of pigment producing and secondary metabolites producing ability of actinomycetes so the exploration of unexplored regions of Chambal territory and some other parts of Madhya Pradesh is necessary to explore some novel actinomycetes. The present study, focus on novel actinomycetes, which have diffusible pigment ability in media and antimicrobial abilities against various pathogenic microorganisms. This pigment producing ability of actinomycetes could be used to extract natural pigment and useful to various pharma, food and textile industries to replace synthetic colours.

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2. Methods

2.1 Isolation & screening of pigment producing actinomycete

Soil samples were collected from different sites of Chambal territory of Madhya Pradesh (MP) regions, India. Soil collected from 8-10 cm depth of surface in a sterilized polythene bags and brought to the laboratory for further experiment [7]. One gram soil sample was mixed in sterile distilled water and allowed for shaking in rotatory shaker for 10 minutes and serially diluted. Samples were spread over surface of plates using different media as Starch Casein Agar (SCA), Actinomycetes Isolation Agar (AIA), Yeast Extract-Malt Extract Agar (YEMA, Inorganic Starch-Salt agar (ISSA), Nutrient agar (NA), Starch Agar (SA) media and Peptone Yeast Iron Agar (PYIA) media and incubated for 6-7 days at 30°C temperature. The isolated actinomycetes cultures were further purified on respective media and stored in BOD incubator [8,9]. Among 85 actinomycete isolates only five actinomycetes showed pigment producing ability but on the basis of diffusion ability only one actinomycete ARITM02 was selected for further study which also possessed antimicrobial activities. Pigment producing ability of selected isolate ARITM02 was tested on solid as well as in broth media.

2.2 Characterization of actinomycete isolate

The morphology of selected isolate AR-ITM02 was found spore bearing hyphae with entire spore chain along with substrate and areal mycelium under light & scanning electron microscope [10], and grouped into three types viz., Flexible- Rectiflexibiles (RF), Open loops- Retinaculiaperti (RA) and Spira- Spirales (S). A characteristic of the spore bearing hyphae and spore chains were determined by the direct microscopic examination of the culture. Adequate magnification used to establish the presence or absence of spore chains. Different biochemical tests as catalase reduction, nitrate reduction, H_2S production, starch hydrolysis, casein hydrolysis, citrate utilization, Indole, MR-VP, gelatin hydrolysis and utilization of different sugars were performed [11].

2.3 Phylogenetic analysis

The selected isolate ARITM02 was subjected for 16s rRNA molecular sequencing and the genomic DNA was isolated, extracted and amplified using high fidelity PCR. PCR product was sequenced bi-directionally using the 16s primers. The 16S rRNA gene fragment was amplified using universal primers (forward primer -5'-GCCTAACACATGCTGG-5' and reverse primer -5'-GTATTACCGCGGCTGCTGG-5') [12] analyzed by performing BLAST search tool. The MEGA 5.0 version was used for phylogenetic and molecular evolutionary analysis [12, 13].

2.4 Antibiogram of selected isolate activity

The selected isolate ARITM02 was also tested for antimicrobial activity against different bacterial and fungus cultures by over lay agar method and well agar diffusion method [14]. The bacterial cultures as Escherichia coli (MTCC 40), Pseudomonas aeruginosa (MTCC 8165), Staphylococcus aureus (MTCC 3160), Bacillus subtilis (MTCC 1134), Enterobacter aerogenes (MTCC 7325), Bacillus cereus (MTCC 1307), Proteus vulgaris (MTCC 1771) and fungi as Aspergillus niger (MTCC 9651), Aspergillus fumigatus (MTCC 2551), Candida albicans (MTCC3017), Microsporum canis (MTCC 2820), Microsporum fulvum (MTCC 2837) and Trichophyton rubrum (MTCC 296) were tested.

3. Results

3.1 Isolation of actinomycetes

Total 85 actinomycetes were isolated from different sites of Chambal territory and Madhya Pradesh regions such as playground soil of ITM University, medicinal garden of ITM University, Poultry farm, Agriculture field of Morena, Chambal Ravine area, forest soil of Shivpuri, agriculture field of Bhopal, sitholi campus of ITM University (Fig. 1). Maximum positive samples were found from Medicinal garden of Madhya Pradesh. Different ISP media were used for isolation of actinomycetes but Starch Casein Agar (SCA) media was found most suitable for growth of actinomycetes (Table 1). The pigment producing actinomycete AR-ITM02 was selected on the basis of its pigment diffusion ability in broth and solid media. The results showed that the selected isolate showed good growth with white aerial mycelium, wine red substrate mycelium, and diffusible pigment producing ability on starch casein agar media only.

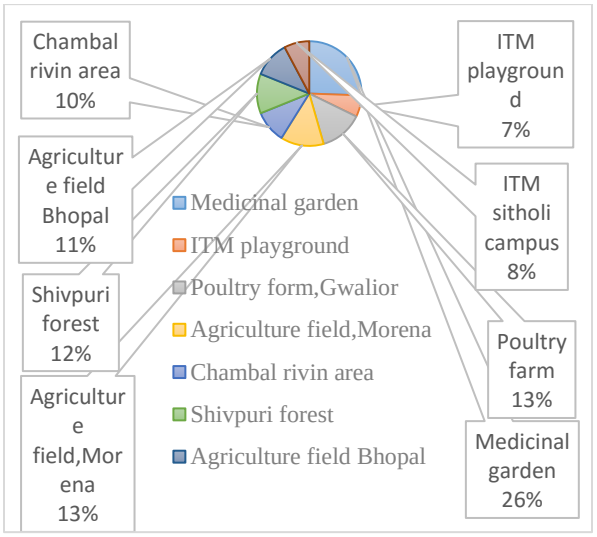

Fig. 1: Percentage distribution of isolated actinomycetes, from different soil samples at Madhya Pradesh regions.

3.2 Identification and characterization of selected isolate (ARITM02)

The dark wine red color pigment was produced on starch casein agar. The isolate utilized various carbon sources for growth as glucose, arabinose, xylose, mannose and fructose for its growth, while sucrose, rhamnose and rhafinnose and were not utilized. Degradation of starch, casein and citrate were observed but the selected isolate does not degrade gelatin (Table 2 & 3).

Light microscopic examination showed gram positive dichotomously branched spore chains, whereas smooth spore surface was visualized by scanning electron microscope. Scanning electron microscope showed smooth spore surface. The spore chains were in spiral form with 10 to 20 spores or more per chain (Fig. 2,3). Morphological,

physiological and biochemical characteristics revealed that ARITM02 is similar to Streptomyces, according to the Bergey's Manual of Determinative Bacteriology [15]. The isolate ARITM02 was found aerobic, Gram positive, non acid fast. The isolate is susceptible to streptomycin (10 µg/mL). Growth was best observed on starch casein agar medium at 30°C temperature

Table 1: Cultural characteristics of the selected isolate ARITM02 on different International Strptomyces Project (ISP) media

Media used	**Growth**	**Aerial mycelium**	**Substrate mycelium**	**Soluble pigment**
Yeast extract-Malt extract	Good	White	Yellow	None
Oatmeal agar (ISP-3)	Good	Grey	Yellow	None
norganic Starch-Salt agar	Good	Grey	Yellow	None
Glycerol asparagines agar	Good	Grey	Yellow	None
Peptone yeast extract iron agar	Good	Grey	Yellow	None
Starch agar	Good	Grey	Yellow	None
Starch casein agar	Excellent	White	Red	Yes

Table 2: Biochemical Characteristics of the actinomycete ARITM02

Physiological tests	**Reaction**
Citrate utilization	+ve
H_2S production	- ve
Gelatin	- ve
Nitrate reduction	- ve
Catalase production	- ve
Starch hydrolysis	-ve
Indole	+ ve
Casein hydrolysis	- ve
Methyl red (MR)	+ ve
Voges-proskauer (VP)	- ve
Catalase	- ve
Glucose	AG
Xylose	G
Fructose	AG
Maltose	AG
Rhamnose	G
Sucrose	AG
Raffinose	G
Ribose	G
Galactose	AG
Maltose	AG

A- Acid production, G- Gas production, +ve = Positive, -ve = Negative

3.3 Molecular identification and phylogenic analysis

The 16S rRNA gene sequence of Streptomyces sp. ARITM02 was deposited at National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences from strains closely related to Streptomyces sp. were retrieved from the GeneBank database using Basic Local Alignment Search Tool (BLAST) [16]. The Phylogenetic tree in Figure 4 shown that Streptomyces flavofucus ARITM02 formed a close distinct line with clade encompassed by Streptomyces flavofuscus strain.

3.4 Antibiogram of isolate Streptomyces flavofucus ARITM02

The antibiogram of the isolate indicate shown good antibacterial activity against S. aureus, B. cereus, E. coli, B. subtilis, E. aerogenes, P. aeruginosa and P. vulgaris. The isolate also has good antifungal activity against C. albicans and A. niger but did not show any activity against skin pathogens (dermatophytes). The isolate has broad spectrum activity against test bacterial cultures (Fig. 5).

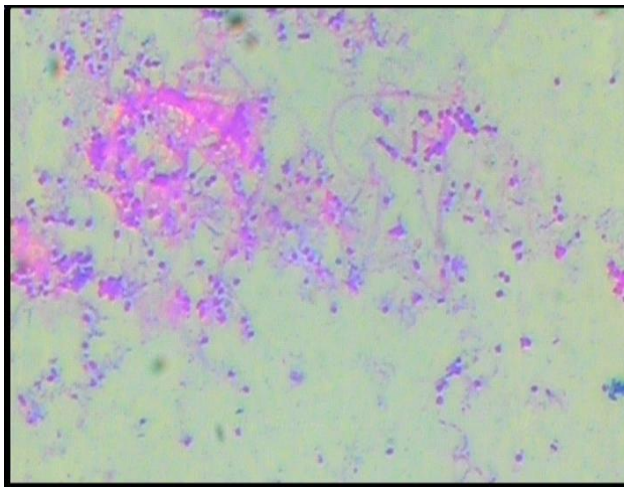

Fig. 2 Spore chains of the selected isolate

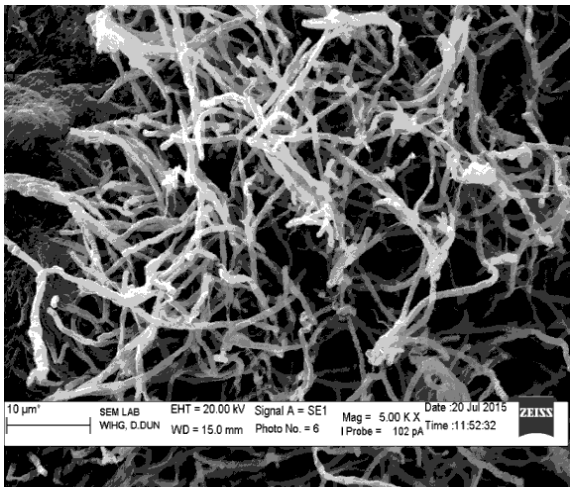

Fig. 3 Spore view of the selected isolate under light microscope from 7 days scanning electron microscope from 14 days old culture (× 400), Culture (× 5,000).

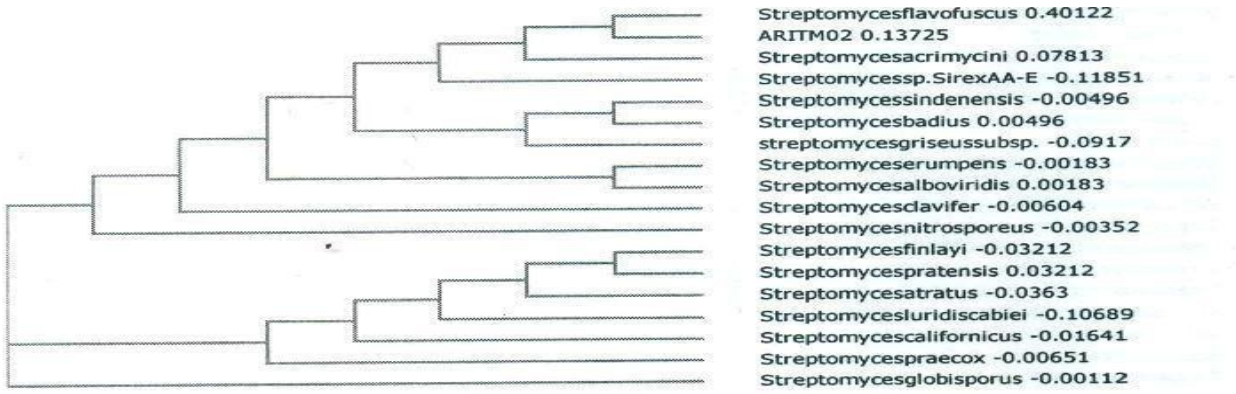

Fig.4 Phylogenic tree based on 16S rRNA gene sequence showing relationship between AR-ITM02 and related members of the genus Streptomyces.

Table 3: Antibiogram of selected isolate ARITM02 by agar overlay method against test pathogens**.**

Test pathogens	**Zone of inhibition**
S. aureus	22
B. subtilis	22
B. cereus	13
E. coil	24
P. aeruginosa	18
E. aerogenes	18
P. vulgaris	00
A. niger	16
A. fumigates	00
C. albicans	18
M. canis	00
M. fulvum	00
T. rubrum	00

4. Discussions

The actinomycetes have shown their importance biotechnologically and industrially. The isolation and characterization of actinomycetes is an important approach for industrially important natural colors [17]. For discovery of novel, potent and industrially beneficial, actinomycetes have been intensively searched from past few decades [18]. Actinomycetes are free living, gram positive bacteria found widely distributed in soil, water and plants. Actinomycetes has been identified as one of the major group of soil population which may vary with the soil type, in present

study the soil samples were collected from rhizosphere soil of Chambal territory and many other parts of Madhya Pradesh regions. The soil of Chambal territory is similar with desert soil [19]. The collection of soil samples and isolation of actinomycetes from desert soil were also supported by Selvameenal et al, (2008) who confirmed the presence of potential actinomycetes with pigment producing ability along with antimicrobial activities of actinomycetes. Several researchers have also reported the actinomycetes, isolated from desert soil [20-22]. It is reported that rhizosphere soil is rich resource of microorganisms including actinomycetes with large population which produces many bioactive compounds. The actinomycetes populations are very common in rhizosphere soil and found widely in plant root systems [23-25]. Many colours are routinely used in medicine, pharma industries, and cosmetics production and these pigments are produced by a wide variety of microorganisms including several species of bacteria and fungi, similarly the results of our study indicates the antimicrobial activities and natural pigment producing ability of actinomycete which might be useful in many industries [26]. Mostly pigments are reported as a common substance produced by animals, plants and microorganisms. These pigments of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black [27]. In the present study, the actinomycetes were isolated from soil samples and then pigment producing actinomycete was purified in different International Streptomyce Project (ISP) media. The selection of pigment producing actinomycete was done on the basis of incubation time and diffusion ability of pigment in media. Gram staining was performed for morphological view which conformed the spore chain formation of actinomycete as Sembiring et al. (2000) suggested previously [28] and the scanning electron microscopy confirms the smooth spore chain formation colonies of actinomycete according to Kokare et al. (2004) [29]. Seven different media were used, but Starch casein agar showed excellent growth and pigment diffusion ability of Streptomyces flavofucus ARITM02 sp, it may be due to enough amount of nutrient available in media. It is also noted that isolate showed pigment producing ability on Starch casein agar only. The 16s rRNA sequencing confirms that the actinomycete belongs to Streptomyces species. Streptomyces shared almost 80% of total antibiotic production as compared to other genera of actinomycetes [30]. The actinomycete also has antimicrobial activities including bacteria, fungus, yeast etc. It is observed that Streptomyces flavofucus ARITM02 sp. have excellent ability to inhibit both Gram positive and Gram negative bacteria and showed noticeable zone of inhibition but it is not showing any activity against dermatophytes. Porter et al reported that almost all actinomycetes have antimicrobial properties if proper culture conditions provided [31].

5. Conclusions

It is concluded that Streptomyces flavofucus ARITM02 belongs from Streptomyces sp. which also has diffusible pigment production ability along with good antimicrobial. It could be open a door for food processing industry as additives colorful beverages, in pharmaceutical industries, textile industries as a natural colorant and might be useful in cosmetic industries if the toxicity tests found negative.

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