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Research Article

ISOLATION AND SCREENING OF ANTIMICROBIAL AND EXTRACELLULAR PIGMENT-PRODUCING ACTINOMYCETES FROM CHAMBAL TERRITORY OF MADYA PRADESH REGION, INDIA

RAMENDRA SINGH PARMAR[1], CHARU SINGH[1], PRAGATI SAINI[2], AJAY KUMAR[1]*

[1]Department of Life Sciences, ITM University, Gwalior, Madhya Pradesh, India. [2]Department of Microbiology, KRG PG College, Gwalior, Madhya Pradesh, India. Email: kumarajayitm@gmail.com

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ABSTRACT

Objective: In the present study, the objective is to isolate, characterize, and study of biological activity of pigment-producing actinomycetes.

Methods: 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 International Streptomycetes Project 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 overlay agar method and well agar diffusion method against various pathogenic microorganisms.

Results: Among 85 actinomycetes isolates, only AR-ITM02 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 has also shown notable antimicrobial activities against various pathogens.

Conclusion: These significant results make Streptomyces suitable for further investigation and industrial exploitation. The 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.

Keywords: Actinomycetes, Pathogenic microorganisms, Pigments, Scanning electron microscopy.

INTRODUCTION

Actinomycetes are Gram-positive bacteria and they are 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 toward 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, and 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, and researchers 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, colors have been widely applied in many areas such as foods, clothes, paintings, cosmetics, pharmaceuticals, and plastics and they also play an important role in food colorant. Pigments have interesting role in industry for its nanotechnology as it used in bioplastics 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 22,500 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 such as 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 focuses on novel actinomycetes which have diffusible pigment ability in media and antimicrobial abilities against various pathogenic microorganisms.

METHODS

Isolation and screening of pigment-producing actinomycete

Soil samples were collected from different sites of Chambal territory of Madhya Pradesh regions, India. Soil collected from 8 cm to 10 cm depth of surface in a sterilized polyethene bags and brought to the laboratory for further experiment [7]. 1 g 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, yeast extract-malt extract agar, inorganic starch-salt agar, nutrient agar, starch agar media, and peptone yeast iron agar media and incubated for 6-7 days at 30°C temperature. The isolated actinomycetes culture were further purified on respective fresh media and stored in biochemical oxygen demand incubator [8,9]. Among 85 actinomycete isolates, only five actinomycetes showed pigment-producing ability; however, on the basis of diffusion ability, only one actinomycete AR-ITM02 was selected for further study which also possessed antimicrobial activities. Pigment-producing ability of selected isolate AR-ITM02 was tested on solid as well as in broth media.

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 and scanning electron microscope [10] and grouped into 3 types, *viz*., flexible-rectiflexibiles, open loops-retinaculiaperti, and spira-spirales(S). A characteristic of the spore-bearing hyphae and spore chains was 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, methyl red and voges - proskauer, gelatin hydrolysis, and utilization of different sugars were performed [11].

Phylogenetic analysis

The selected isolate AR-ITM02 was subjected to 16s rRNA molecular sequencing, and the genomic DNA was isolated, extracted, and amplified using high-fidelity polymerase chain reaction (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 basic local alignment search tool (BLAST). The MEGA 5.0 version was used for phylogenetic and molecular evolutionary analysis [12,13].

Antibiogram of selected isolate activity

The selected isolate AR-ITM02 was also tested for antimicrobial activity against different bacterial and fungus cultures by overlay agar method and well agar diffusion method [14]. The bacterial cultures as *Escherichia coli* microbial type culture collection (MTCC 40), *Pseudomonas aeruginosa* (MTCC 8165), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 1134), *Enterobacter aerogenes* (MTCC 7325), *Bacillus cereus* (MTCC 1307), and *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.

RESULTS

Isolation of actinomycetes

A total of 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 International Streptomycetes Project (ISP) media were used for isolation of actinomycetes, but SCA media were 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 SCA media only.

Table 1: Cultural characteristics of the selected isolate AR-ITM02 on different 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
Inorganic 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

ISP: International Streptomycetes project, YEME: Yeast extract-malt extract

Identification and characterization of selected isolate (AR-ITM02)

The dark wine red color pigment was produced on SCA. The isolate utilized various carbon sources for growth as glucose, arabinose, xylose, mannose, and fructose for its growth while sucrose, rhamnose, and raffinose were not utilized. Degradation of starch, casein, and citrate were observed, but the selected isolate does not degrade gelatin (Tables 2 and 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 20 spores or more per chain (Figs. 2 and 3). Morphological, physiological, and biochemical characteristics revealed that AR-ITM02 is similar to Streptomyces according to the Bergey's manual of determinative bacteriology [15]. The isolate AR-ITM02 was found aerobic, Gram-positive, non-acid fast. The isolate is susceptible to streptomycin (10 µg/mL). Growth was best observed on SCA medium at 30°C temperature.

Molecular identification and phylogenic analysis

The 16S rRNA gene sequence of Streptomyces sp. AR-ITM02 was deposited at NCBI. The 16S rRNA gene sequences from strains closely related to Streptomyces sp. were retrieved from the GeneBank

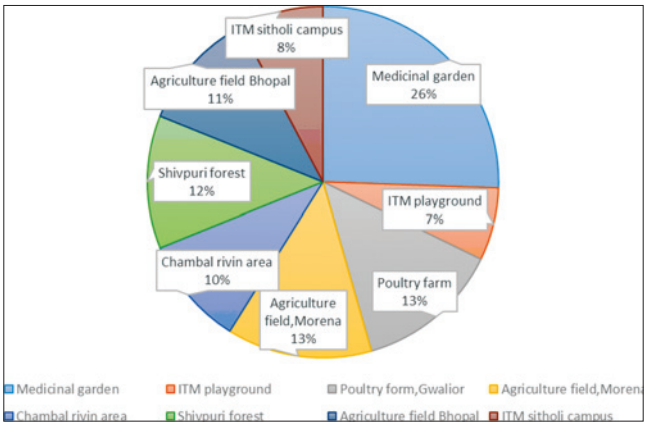

Fig. 1: Percentage distribution of isolated actinomycetes from different soil samples at Madhya Pradesh regions

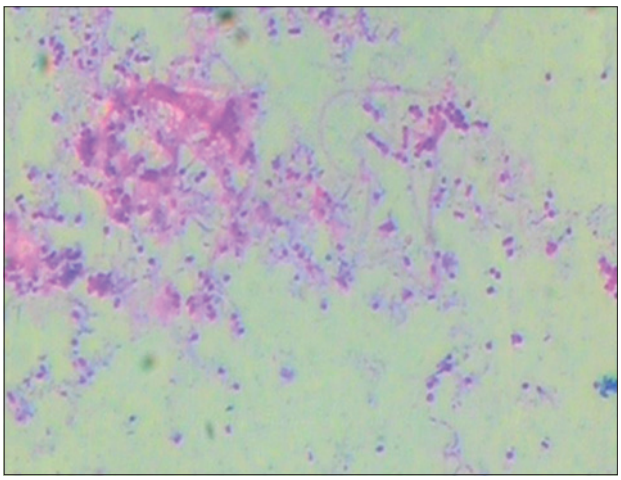

Fig. 2: Spore chains of the selected isolate isolate under-examined by light microscope from 7 days old culture on starch casein agar (×400)

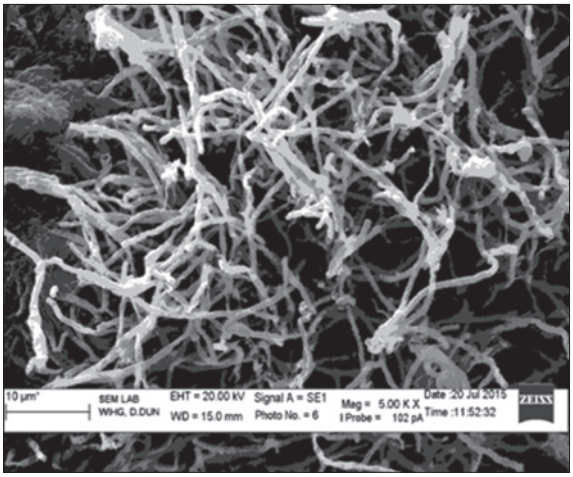

Fig. 3: Spore view of the selected isolate under scanning electron microscope from 14 days old culture on starch casein agar (×5000)

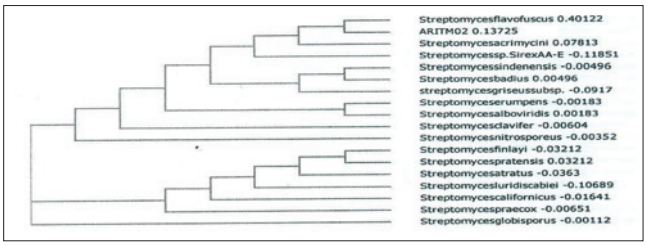

Fig. 4: Phylogenic tree based on 16S rRNA gene sequence showing relationship between AR-ITM02 and related members of the genus Streptomyces

Table 2: Biochemical characteristics of the actinomycete AR-ITM02

Physiological tests	Reaction
Citrate utilization	+ve
H_2S production	+ve
H_2S production	-ve
Gelatine	-ve
Nitrate reduction	-ve
Catalase	-ve
Starch	-ve
Indole	+ve
Casein	-ve
MR	+ve
VP	-ve
Catalase	-ve

+ve: Positive, -ve: Negative

Table 3: Utilization of different carbon sources by the selected actinomycete AR-ITM02

Source	Utilization	Source	Utilization
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

database using BLAST [16]. The phylogenetic tree in Fig. 4 showed that Streptomyces AR-ITM02 formed a close distinct line with clade encompassed by *Streptomyces flavofuscus* strain.

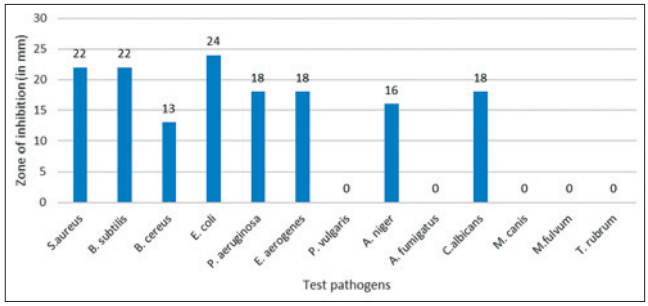

Fig. 5: Antibiogram of selected isolate AR-ITM02 by well agar diffusion method against test pathogens

Antibiogram of isolate Streptomyces AR-ITM02

The antibiogram of the AR-ITM02 isolate indicate that AR-ITM02 isolate has 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).

DISCUSSION

The actinomycetes have shown their importance biotechnologically and industrially. The isolation and characterization of actinomycetes are an important approach to industrially important natural colors [17]. For discovery of novel, potent, and industrially beneficial, actinomycetes have been intensively searched from the past few decades [18]. Actinomycetes are free living, Gram-positive bacteria found widely distributed in soil, water, and plants. Actinomycetes have been identified as one of the major groups of soil population which may vary with the soil type; in the 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 to desert soil [19]. The collection of soil samples and isolation of actinomycetes from desert soil were also supported by Selvameenal *et al.*, which 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 colors 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 indicate 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 actinomycetes were purified in different ISP media. The selection of pigment-producing actinomycete was done on the basis of incubation time and diffusion ability of pigment in media. The Gram staining was performed for morphological view which confirmed the spore chain formation of actinomycete as Sembiring *et al.* suggested previously [28] and the scanning electron microscopy confirmed the smooth spore chain formation colonies of actinomycete according to Kokare *et al.* [29]. Seven different media were used, but SCA showed excellent growth and pigment diffusion ability of AR-ITM02; it may be due to enough amount of nutrient available in media. It is also noted that AR-ITM02 showed pigment-producing ability on SCA only. The 16s rRNA sequencing confirms that the actinomycete AR-ITM02 belongs from Streptomyces species. Streptomyces shared almost 80% of total

antibiotic production as compared to other genera of actinomycetes as our study also suggested which confirmed the Streptomyces [30]. The actinomycete also has antimicrobial activities including bacteria, fungus, and yeast. It is observed that Streptomyces AR-ITM02 had excellent ability to inhibit both Gram-positive and Gram-negative bacteria and showed noticeable zone of inhibition, but it does not show any activity against dermatophytes. Porter *et al.* reported that almost all actinomycetes have antimicrobial properties if proper culture conditions provided [31]. The antibiogram showed large zone of inhibition against *S. aurious, B. subtilis,* and *E. coli* which are also conclude the excellent potency of Streptomyces as Kumar *et al.* reported in their studies.

CONCLUSION

It is concluded that it belongs from Streptomyces which also have diffusible pigment-production ability. 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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