Streptomyces sp D25 Isolated from Thar Desert Soil, Rajasthan producing Pigmented Antituberculosis Compound only in Solid Culture

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While screening actinomycete isolates for anti-TB natural products actinomycete strain D25, isolated from Thar Desert soil, Rajasthan, was observed to produce yellow-orange pigmented anti-TB compound in solid culture. The effect of medium co and nutrient concentration on the growth and anti-TB pigment production by the strain D25 was studied. Further, the effect of mechanical shaking and that of the producing organism itself on the yellow orange pigment production was studied by submerged fermentation. Strain D25 was found to produce the bioactive yellow-orange pigment only on solid culture but not in liquid culture in which the pigment got degraded either by mechanical shaking or by the enzyme action of the producing strain D25 itself. Effect of medium consistency and nutrient concentration was studied by using different concentrations of YEME broth and YEME agar. Medium consistency and filamentous mycelial structure did not have significant effect on bioactive pigment production by the strain D25 even while using different nutrient concentrations.

Key words: Actinomycetes, Streptomyces, Thar Desert, solid culture, Antituberculous.

Actinomycetes, in particular streptomycetes, have a filamentous growth habit adopted for surface growth in solid cultures in nature[1]. However, our understanding of the physiology of these organisms is largely based on their growth in submerged liquid cultures. According to the literature, submerged fermentation followed by extraction of the metabolites from the culture filtrate is the mostly used current procedure, probably due to the easy accessibility of solubilized compounds in the culture filtrate. However, some disadvantages are apparent. Antibiotics like fumaridamycin were detected with much difficulty in submerged cultures because the mycelium of the producing strain inactivated the antibiotic more readily than in agar culture[2]. In addition, actinomycetes are often found to produce antibiotics only on solid media[3,4,5,6,7]. But very little is known as to why such activity is restricted and not detected in submerged cultures[3,4].

Production of a majority of industrially important secondary metabolites from actinomycetes is carried out using submerged fermentation processes where they exhibit diverse morphological forms[8,9]. Morphology is influenced by environmental conditions such as medium composition and shear stress[10,11]. Further, morphology and product formation have been observed to be closely related[12]. For eg. morphology and avermectin production by

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Streptomyces avermetilis were influenced by factors such as nitrogen source, dissolved oxygen level and inoculum volume[13]. Pellets of small size and high density were found to promote avermectin production. In a study by Shomura et al.,[3] the antibiotic production of *Streptomyces halstedii,* which showed activity against gram negative bacteria only in agar dishes, was well correlated with its mycelial morphology. The vegetative mycelium was filamentous in antibiotic producing agar cultures, but fragmented in non-producing submerged cultures. In this study, maintaining submerged cells filamentous by using diluted media, production of antibiotic in the submerged fermentations was accomplished.

While screening actinomycetes for anti TB activity, actinomycete strain D25, isolated from Thar Desert soil Rajasthan, was found to produce an antimycobacterial yellow-orange pigment only in agar medium but not in liquid culture. Based on the above literature, an attempt was made to study the effects of medium consistency and nutrient concentration on yellow-orange pigment production by the actinomycete strain D25.

MATERIALS AND METHODS

Description of actinomycete strain D25

Actinomycete strain D25 was isolated from Thar desert soil, Rajasthan in the year 2005 for the isolation of antimicrobial compounds. Crude yellow-orange pigment produced by the strain D25 showed promising activity against methicillin and vancomycin resistant *S. aureus* as well as the multi drug resistant and extensively drug resistant strains of *M. tuberculosis*. Viability of strains D25 is maintained in yeast extract malt extract agar[14] as well as in 30 % glycerol broth.

Production of pigment and confirmation of its activity

Strain D25 was inoculated on to YEME agar plates as well as in 100 ml of YEME broth in 500 ml flask. Plates were incubated at 28^0C for 10 days whereas the culture flask was incubated in rotary shaker. After scrapping the mycelial growth, the secreted yellow orange pigment from the agar medium was extracted using ethyl acetate[15]. Yellow-orange pigment, if any, from YEME broth was extracted overnight using equal volume of ethyl acetate and concentrated using rotary evaporator. Antibacterial activity was confirmed by disc diffusion method against *S. aureus* MTCC96 and clinical isolate of *S. aureus* resistant to methicillin and vancomycin. Antimycobacterial activity was confirmed by adapting luciferase reporter phage (LRP) assay using standard strain *M. tuberculosis* H37Rv at 100 µg/ml concentration[16].

Stability of pigment during submerged fermentation

Since the strain D25 failed to yield yellow-orange pigment in liquid culture, an experiment was conducted to determine whether the pigment was produced and got degraded during submerged fermentation either by the producing strain itself or by mechanical shearing. Each 25 ml of YEME broth was prepared in three 250 ml conical flasks and sterilized by autoclaving. About 2.5 mg of crude yellow-orange pigment extracted from YEME agar inoculated with the strain D25 was added into the second and third flasks. Then 2.5 ml broth culture of strain D25 was inoculated into first and third flasks. All the flasks were kept in rotary shaker at 120 rpm for 10 days[3]. The second flask was used to determine the effect of agitation on the yellow-orange pigment. The third flask was used to determine the effect of actinomycete strain D25 on the yellow-orange pigment.

After 10 days of incubation, the culture broths from all the flasks were tested for antimicrobial activity against *S. aureus* MTCC96 by agar well diffusion method. Pigments from all the three flasks were also extracted using ethyl acetate and subsequently tested for antimicrobial activity by disc diffusion method. Activity against *M. tuberculosis* H37Rv was tested by LRP assay.

Effect of solid and liquid media on growth, micromorphology, pigment production and bioactivity of the strain D25

YEME agar (30ml/plate) and YEME broth (50ml/flask) were prepared at five different concentrations viz., 2X, 1X, 1/2X, 1/4X and 1/10X. Three hundred microlitres of inoculum were transferred to each of YEME agar plates and 500 µl of inoculum was transferred to YEME broth. All the plates were incubated at 28^0C and all the flasks were kept in rotary shaker at 28^0C at 90 rpm. For every 24 hours, growth rate and pigment production were visually observed. Mycelial structure was observed under bright field microscope at 10X magnification[3] (modified). Agar

plugs from YEME agar and the cell free supernatants from YEME broth cultures were tested against *S. aureus* MTCC96 by agar plug method and well diffusion method, respectively. The crude pigment from YEME broth and YEME agar was extracted using ethyl acetate. The concentrated crude extracts were tested against *M. tuberculosis* H37Rv.

RESULTS AND DISCUSSION

Strain D25 grows well both on YEME agar and YEME broth but yellow-orange pigment production was observed only on agar culture. While testing the activity, only the crude extract prepared from YEME agar showed 14 mm zone of inhibition against *S. aureus* MTCC96 and 84.12% reduction in relative light unit (RLU) against *M. tuberculosis* H37Rv. The cell free supernatant from YEME broth failed to inhibit both the test organisms. This indicated that the bioactive pigment responsible for the inhibitory activity was not produced in liquid culture at all. Previously many authors reported that actinomycetes were often found to produce antibiotics only on solid media[5,6,7].

The yellow-orange pigment of strain D25 inoculated in YEME broth of flask II and III could be extracted in ethyl acetate. But no yellow-orange pigment could be extracted from the first flask which contained YEME broth inoculated with the strain D25. In well diffusion and disc diffusion methods, the cell free supernatant and the ethyl acetate extract obtained from the flask I showed no activity indicating the absence of the yellow-orange pigment in submerged culture. In well

Table 1. Effect of submerged fermentation of strain D25 on antibacterial and antimycobacterial activity

Reaction flasks	Test organisms		
	S. aureus MTCC 96 (Zone of inhibition in mm in dm)		*M. tuberculosis* H37Rv (% reduction in RLU)
	Well diffusion	Disc diffusion	
I	-	-	0
II	22	14	88.7
III	23	14	84.1

Flask I – YEME broth + Strain D25 culture; Flask II – YEME broth + yellow-orange pigment; Flask III – YEME broth + yellow pigment + Strain D25 culture; - absence of inhibition zone; 0 – not tested

Table 2. Antibacterial and antimycobacterial activity of strain D25 grown on different concentration of YEME agar

Incubation period	Antibacterial activity (Zone of inhibition in mm in dm)					Antimycobacterial activity (% reduction in RLU)				
(Days)	2X	1X	1/2X	1/4X	1/10X	2X	1X	1/2X	1/4X	1/10X
1	0	0	0	0	0	-	-	-	-	-
2	0	18	20	21	21	31.32	67.74	83.50	48.43	44.02
3	0	20	20	21	20	95.87	98.67	99.27	97.12	99.28
4	0	20	22	22	20	89.47	99.19	99.30	92.56	98.67
5	11	19	20	19	15	98.58	99.16	99.16	99.25	96.05
6	12	19	20	19	15	99.04	99.31	99.15	99.23	91.53
7	16	20	19	19	15	99.29	99.08	99.29	99.23	96.66
8	16	20	19	19	15	98.90	99.31	99.26	96.83	98.65
9	19	20	20	20	16	99.27	99.22	99.27	85.49	87.76
10	21	21	20	20	16	99.04	99.31	99.26	96.21	98.62

0 – no inhibition; - - not tested

diffusion and disc diffusion methods, the cell free supernatant and its ethyl acetate extract obtained from the flask II and III showed activity against *S. aureus* MTCC96 (Table 1). In an earlier study by Shomura et al.,[3], about 1300 isolates of 6500 actinomycetes tested showed antimicrobial activity against one or more of the organisms tested by agar plug method. In the secondary screening, about 25 (1.9%) of the 1300 strains were found to be non-producers in submerged cultures. Reports on antibiotics isolated only from agar cultures of actinomycetes are rare. According to Mayurama et al., [2], fumaridamycin was detected with difficulty in submerged cultures because the mycelium of the producing strain inactivated the antibiotic more readily than in agar culture. The shake flask experiment was carried out to test whether such inactivation is happening with D25; it was evidenced that the bioactive yellow pigment production was neither affected by mechanical shearing nor by the strain D25 itself.

Growth of D25 strain was observed on all the concentrations of YEME agar. The development of aerial mycelium and sporulation started earlier in low concentration (1/10X) of YEME agar compared to high concentration (2X YEME agar). The yellow pigment production was observed on all the five concentrations of YEME agar medium. The pigment production was fast in low nutrient medium (1/10X) compared to high nutrient medium (2X). In YEME broth also growth was observed from the first day of incubation. In high (2X) and low (1/10X) concentrations of YEME broth, granule and thread like growths were observed, respectively. No pigment production was observed in all the five concentrations of YEME broth even after 10 days of incubation.

The mycelial structure of strain D25 was fragmented when grown in high concentrations of YEME broth whereas it was filamentous in low concentrations. In YEME agar, no fragmentation was observed when the strain D25 was grown on all the five concentrations. However, it produced long substrate mycelium in low concentration of YEME agar. The agar plugs from all four concentrations of YEME agar, except from 2X inoculated with strain D25 showed activity against *S. aureus* MTCC96. Only the agar plug from 2X concentration of YEME agar taken on the 5th day of incubation showed inhibition. The cell free supernatant from all the five concentration of YEME broth failed to inhibit *S. aureus* MTCC 96 when tested by agar well diffusion method. The ethyl acetate extract of strain D25 grown on 2X, 1/4X and 1/10X concentrations of YEME agar prepared on 3rd day of incubation was found to inhibit *M. tuberculosis* H37Rv. The ethyl acetate extracts from 1X and 1/2X YEME agar prepared after 2 days of incubation was found to inhibit *M. tuberculosis* H37Rv. However, the cell free supernatants from all these failed to inhibit *M. tuberculosis* H37Rv (Table 2). Similar to the observation of Shomura et al.,[3] the bioactive pigment production by the strain D25 was seen only in agar culture where the vegetative mycelium was filamentous. By decreasing the concentration of YEME broth from 2X to 1/10X the mycelial filamentation was found to increase. However, none of the five concentrations of YEME broth tested supported pigment production. This observation evidenced that the filamentous mycelial structure does not have a significant effect on bioactive pigment production by the strain D25 while the filamentous mycelium and bioactive pigment production was observed in all the concentrations of YEME agar. Ohnishi et al.,[17] reported a factor dependent 2-aminophenoxazin-3-one containing yellow pigments, Grixazone A and B, produced under phosphate depletion by *Streptomyces griseus*. Similarly, it was assumed that, optimizing the medium components may trigger the pigmented bioactive compound production by the strain D25 in liquid cultures.

Based on the above observations, the present study concludes that solid state fermentation is the only approach for the production of antituberculosis pigment from the actinomycete strain D25. Further, these observations indicated the uniqueness of the strain D25 in terms of fermentation and bioactive metabolite production.

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