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EFFECT OF PH IN THE NATURE OF PIGMENTATION AMONG ACTINOMYCETES

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ABSTRACT
Actinomycetes are found to be prominent microbes among the micro organisms communities. About 70% of antibiotics have been discovered from actinomcyetes and these are found to be natural products used in medicinal fields. In this investigation we aimed to isolate the Streptomyces species from unexplored Western Ghats regions of Tamil Nadu to obtain pigmented metabolites of bioactivities and their stability in different pH. Two potential strains have been isolated from the regions and they were characterized morphologically, biochemically and physiologically. From the characterization results these two strains belongs to Streptomyces genus. In order to obtain the pigments from the strains, different media were used to maximize the production status and the pigments were extracted using polar and non polar solvents. The growth and pigment production has been observed in various pH ranges for MDUF1 and TIR. The strain TIR produced green colour pigment in all pH ranging from 3 to 11 whereas the strain MDUF1 grew well from pH range of 3 to 8 but the pigment colour was not uniform in all ranges. The crude pigments in different pH from these two strains were subjected to TLC and good separation of compounds were found in petroleum ether: ethyl acetate combination. Further the pigments were analysed for FT-IR and different ranges were observed in different pH range. Further studies will be focused on finding out the reasons behind the changes in the pigmented compounds due to pH influences.

KEY WORDS
Actinomycetes, antagonism, metabolites, pH, FT-IR.

1. INTRODUCTION

Actinomycetes are the group of gram positive bacteria with high G+C (guanine + cytosine) content which forms branching filaments or hyphae and asexual spores. Actinomycetes had known to produce various kinds of antibiotics and moreover these antibiotics include many pigments. The value of actinomycetes to society in terms of providing useful drugs especially antibiotics and anticancer agents and to the pharmaceutical industry for revenue generating discovery platform is indisputable. It has been shown that the actinomycetes are able to produce many pigments nearly all the primary colors of the rainbow. The pigments maybe dissolved in to the medium or it may be retained in the mycelium. Production of pigments by actinomycetes has been utilized as an important cultural characteristic in describing the organisms. Many of these pigmented metabolites seemed to be bioactive than that of their colorless counterparts. The variations in pH and temperature have definite role on the growth of actinomycetes as well as pigmentation. Since, the pH of the substrate influences the pigmentation of the actinomycetes, this piece of work has been carried out to observe the changes in the pigmented metabolite by altering the pH of the medium.

2. MATERIALS AND METHODS

2.1. Organism used in this study

Two pigmented Actinomycetes strains MDUF1 and TIR which produces pink and bottle green colour respectively had been selected from our culture collection for this study.

2.2. Characterization of organisms

These two strains were characterized through their morphological and biochemical studies. Morphology of spore bearing hyphae with entire spore chain was identified in SCN agar [1] and was observed with a phase contrast microscope by using cover slip method. The cover slip with culture was observed under microscope for the morphology of substrate mycelium and aerial mycelium. The biochemicals tests such as MR-VP, Indole, TSI, Oxidase, Catalase,

Citrase, Estrase, Gelatinase, Caseinase, Urease, Acid from carbohydrates and Nitrate reduction were performed to characterize the biochemical property using the methods of Shirling and Gottlieb [2] with minor modifications.

2.3. Measurement of biomass

One hundred millilitres of various broths were autoclaved, inoculated with 5%inoculums and incubated at room temperature for 14 days in shaking condition. Biomass was harvested after 14 days incubation. The biomass was separated and washed twice with distilled water over pre weighed Whatman No.1 filter paper. The filter paper with fresh biomass was dried in hot air oven at 80ºC for eight hours and cooled in desiccators before weighing for dry cell weight(X). The results were expressed in gram per litre.

2.4. Evaluation of pigment production

The production of pigment was evaluated on the basis of the following parameters; total absorbance of intra and extra cellular pigments (Abs_T); yield factor of pigments on cell growth($Y_{p/x}$). $Abs_T = Abs_{extra} + Abs_{intra}$; $Abs_{intra} = Abs_{extract} \times D$; D=50 V and $Y_{(p/x)} = \Delta Abs_T / \Delta x$.

(Abs_T=absorbance of extra plus intra cellular pigments (U); Abs_{extra}= extra cellular absorbance (U); Abs_{intra}:=intra cellular absorbance (U); $Abs_{extract}$=absorbance in the extract of cell disruption (U); V=Volume of sample submitted to cell disruption for pigments extraction (mL); X= Cell concentration (gL^{-1}) and $Y_{(p/x)}$ = yield factor of pigments on cell growth (U $L\,g^{-1}$)).

2.4.1. Estimation of intracellular pigment production

One gram of fresh biomass was taken in a beaker. Fifty mL of 70% methanol was added and kept over boiling water bath (60ºC) for 2 hours. The biomass was well ground in a clean glass homogenizer. Then it was centrifuged at 5000xg for 10 min. The absorbance value was scanned ($Abs_{extract}$) from 485 nm to 500 nm using UV-Visible spectrophotometer multiplied by the dilution factor in 70% methanol and referred as Abs_{intra}

2.4.2. Estimation of extracellular pigments production

The culture filtrate was taken in a centrifuge tube and spun down at 5000xg for 10 min to remove debris. The clear supernatant was collected and scanned in a spectrometer from 485 nm to 500 nm and was taken as Abs_{extra}.

2.5. Antimicrobial activity of the pigmented isolates

The antimicrobial activity of the crude filtrate was tested using well diffusion method [3]. Indicator organism such as *staphylococcus aureus, S.epidermis, Enterococcous faecalis, Klebsiella pneumoniae, Proteus sp, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Shigella* sp were used. Overnight culture of these pathogens were swabbed on Nutrient Agar plates in which wells were cut and 50µl of culture filtrate obtained from culture broth was added. They were further incubated for 24 h at 37°C. The activity was determined by measuring the diameter of clear zone.

2.6. Effect of pH on growth and pigmentation

The seed media were prepared and adjusted to various pH such as 3.0, 4.0, 5.0, 6.8, 8.0, 9.0, 10.0, 11.0 and strains were streaked continuously and kept for incubation at 37ºC for seven days to check for the growth and pigmentation of the selected strains. The antimicrobial activity of the pigments produced in different pH ranges was also checked.

2.7. Selection of solvent for extraction of pigments

The pigment from culture filtrate and the biomass of the selected strain was extracted using various solvents of polar and non-polar nature. As many as ten different solvents were attempted viz: petroleum ether, ethyl acetate, acetone, chloroform, isobutyl alcohol, propanol, isoamyl alcohol, dichloro methane, ethanol and methanol. The solvent that extracted the maximum quantity of pigment was selected for the further studies.

2.8. Compound separation using TLC

The extracted pigments from the biomass were allowed to run in the Thin Layer Chromatography for the compound separation [4]. The solvent system and its concentration were determined for the compound separation.

2.9. FTIR analysis for crude pigments

The extracted pigments from biomass were condensed and dried in petri plates. The crude samples were scrapped out and were analyzed using FTIR for the compound variation due pH changes.

3. RESULTS AND DISCUSSION

The strains TIR and MDUF1 were observed for their morphology and they showed growth with profusely branched aerial mycelium and the colonies were regular (Fig 1). Pigmentation of the isolates indicated from 3rd day onwards and pigments were of diffusible in nature. Both the strains showed negative to weakly positive for most of the tests except for Citrate utilization and TSI. Both the strains were grown well in acidic ranges and sparse to moderate growth was observed in alkaline ranges. The strains grow well at 28ºC and 37ºC whereas no growth was observed in

extreme conditions. The antimicrobial activity of the pigmented isolates were checked, the strain MDUF1 showed activity against a maximum number of pathogens than that by TIR (Table. 1). These strains were well grown in SCN agar which was further selected as seed medium (Table. 2).

The growth and pigment production has been observed in various pH ranges for MDUF1 and TIR. It was found that the pH had some influence on the growth and pigmentation of the strains. The strain TIR produced green colour pigment uniformly in all pH ranging from 3 to 11 whereas the strain MDUF1 grew well from pH range of 3 to 8 but the pigment colour was not uniform in all ranges. The pigmentation was pink in neutral pH (Table. 3) and a shade of yellow colour (Table. 3) was observed in acidic pH ranges whereas it was dark pink to cherry red in alkaline pH ranges (Table. 3). Since there was the influence of pH in pigmentation of strain MDUF1, it has been further studied. The pigment from this strain MDUF1 was extracted well with butyl alcohol than that of the other solvents employed.

The TLC had been performed for the compound separation from the pigments produced in various pH ranges such as 3.0, 6.8 and 8.0. Different solvent systems had been employed to find out the better separation. The petroleum ether and ethyl acetate (4:1) was seemed to best for separation and hence it was commonly employed for the extraction of pigments produced in all three pH ranges. The pigmented compounds from crude extract were separated by spotting them in TLC plates. The active fractions were further analysed with FTIR for the change in their functional groups due to the influence of pH.

The spectrum of crude pigment extract at pH 6.8 and 8.0 were similar having aromatic (C=C) and carboxyl (C=O) stretching vibrations, whereas the crude pigment extract of pH 3.0 showed an FTIR spectrum with a stretching at C-H (Alkenes) and Arenes (C=C) regions. A weak bending vibration of Methyl (CH_3) deformation was also noted in the extract obtained at pH 8.0. Apart from this vibration for Ester group was observed in the crude pigment extract obtained at pH 6.8. This suggests that there were changes in the pigments produced under different pH ranges, the further studies may hence required finding out the reasons behind the changes in the pigmented compounds due to pH influences.

Table 1: Antimicrobial activity:

PATHOGENS	ZONE OF INHIBITION (cm)	
	MDUF1	TIR
Staphylococcus aureus	0.8cm	-
Staphylococcus epidermidis	1.4cm	0.8cm
Enterococcous faecalis	-	-
Klebsiella pneumoniae	0.6cm	-
Proteus sp	-	-
Escherichia coli	-	-
Pseudomonas aeruginosa	-	0.6cm
Salmonella typhi	1.4cm	-
Shigella sp	0.8cm	-

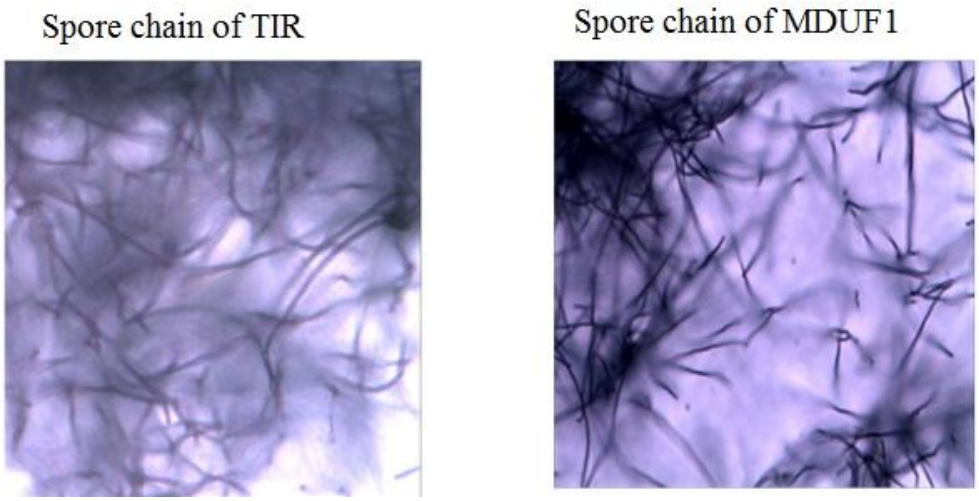
Table 2: Growth of organisms in different media:

MEDIA USED	MAXIMUM YIELD OF MDUF1 (L g^{-1})	MAXIMUM YIELD OF TIR (L g^{-1})
ISP-1	4.52	1.214
ISP-2	0.789	4.125
ISP-3	4.467	0.42
ISP-4	3.327	2.237
ISP-5	10.82	5.389
ISP-6	2.4	3.12
SCA	11.26	9.013
Soybean meal	1.138	3.921
Kuster's	0.704	0.204
Gause inorganic	7.037	6.721

Table 3: Growth of strains TIR and MDUF1 in different pH:

pH	TIR		MDUF1	
	GROWTH	PIGMENTATION	GROWTH	PIGMENTATION
3.0	Well	Green	Well	Yellow
4.0	Mild	Green	Well	Pinkish yellow
5.0	Mild	Green	Well	Pink
6.0	Well	Green	Well	Pink
6.8	Well	Green	Well	Pink
8.0	Well	Green	Well	Cherry red
9.0	Mild growth	Green	Mild	Dark pink
10.0	-	-	-	-
11.0	-	-	-	-

Fig. 1: Morphology of TIR and MDUF1



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