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Research Article

EXTRACTION AND CHARACTERIZATION OF ANTIBACTERIAL PIGMENT FROM *ROSEOMONAS GILARDII* YP1 STRAIN IN YERCAUD SOIL

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ABSTRACT

Objective: The current study is focused on antibacterial pigment production from Yercaud hills region soil bacteria.

Methods: In this present study, a soil sample was collected from a coffee plantation in Yercaud, Salem district. The collected soil sample was used to isolate the pigment-producing bacterial strains. The pigments were extracted by acidified ethanol and there used to screen the antibacterial activity against clinical pathogens. The potential antibacterial pigment producer was identifying and the pigment was characterized by ultraviolet spectroscopy, thin-layer chromatography, and Fourier transform infrared (FT-IR) and also evaluate the minimum inhibitory concentration of pigment against clinical pathogens.

Results: The pigment-producing bacterial strains were isolated from Yercaud soil, among the isolates, YP1 was maximum activity against test pathogens. The potential pigment producer was identified as *Roseomonas gilardii*. The extracted pigments showed the maximum absorbance at 450 nm and their functional groups were identified using FT-IR analysis.

Conclusion: Based on the results, the pigment was extracted from *R. gilardii* and it may serve as potential antibacterial pigment of interest in the food industries, textile industries in natural colorant, and cosmetic industries.

Keywords: Pigment, Roseomonas gilardii, Extraction, Antibacterial activity, Characterization.

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INTRODUCTION

Pigment is a colored compound that presents in the entire living organism in the world, where plants are the principal producers. Pigments are present in leaves, fruits, vegetables, and flowers; they are also found in skin, eyes, and other animal structures and in bacteria and fungi. Pigments have been a well-known pharmacological activity such as anticancer and effective against cardiovascular diseases [1,2]. Through many natural colors are available from ores, insects, plants, and microbes; microbial colorants play a significant role which is food coloring agent, due to its production and easy downstreaming process [3,4]. There are a number of natural pigments but only a few are available quantities for industrial production. Production of pigments from microorganism is beneficial than other sources because microorganisms can grow rapidly which may lead a high productivity of the product [5]. Certain bacteria produce water-soluble pigments which spread through the medium, in which they grow; other give pigments are soluble in fat. The yellow pigment zeaxanthin from *Flavobacterium* species can be used as addictive in poultry feed to fortify the yellow color of the skin of birds or to accentuate the colors of the yolk of the egg and also be used in cosmetic and in food industry. Canthazanthin from the photosynthetic bacterium *Bradyrhizobium* sp. has been used as fish feed for numerous years. *Halobacterium* is also one more source of canthaxanthin, astaxanthin from *Agrobacterium aurantiacum* [6]. The production and application of microbial pigment as natural colorants has been studied by various researchers and is one of the emerging fields of research [7]. Innovations will progress the economy of pigments production by isolating new or creating better microorganisms, by improving the process. Hence, work on the microbial bacterial pigments should be excelled, especially in finding cheap and suitable growth mediums which can reduce the cost and increase its applicability for industrial production [5]. This present investigation has been undertaken to study the antimicrobial activity of microbial pigment isolated from coffee rhizosphere soil sample against clinical pathogens.

METHODS

Collection of sample

The soil samples were collected from rhizosphere of coffee plant area in Yercaud, Tamil Nadu. From all samples, pigment-producing bacteria were isolated and the bacterial cultures were used for further study.

Isolation of pigment-producing bacterial strains

Collected soil samples were used for serial dilution up to 10^{-7} dilutions. A 0.1 ml of aliquots from 10^{-4} to 10^{-6} dilutions were spread on nutrient agar plates and the plates were kept for incubation at 37°C for 48 h. After the incubation, nutrient agar plates were observed for growth and pigment production. Pigment-producing bacterial isolates were used for further study.

Extraction of pigment from pigment-producing bacteria

The pigment-producing bacterial strains were harvested by centrifugation at 6000 rpm for 10 min. Then, the supernatants were discarded and the pellets were resuspended in acidified ethanol (4 ml of 1 M HCL in 96 ml ethanol). Then, the mixture was vortex and the suspension was centrifuged at 6000 rpm for 10 min and the supernatant was collected. The centrifugation was repeated till the pellet changes to colorless. The crude pigments were allowed to acidified ethanol evaporation. After the evaporation of acidified ethanol, dried pigment was collected for further uses [8].

Screening of antibacterial pigment-producing bacteria

An overnight log culture of each pathogenic strain such as *Escherichia coli*, *Klebsiella* sp., *Enterococcus* sp., *Pseudomonas* sp., and *Proteus* sp. was spread evenly on a Mueller-Hinton agar (MHA) plate by swab. Wells were

made on the MHA plates using a gel puncture. After that, the extracted crude pigment (100 µl) was added into each well, and the plates were incubated at 37°C for 24 h. After incubation, the zones of inhibition were measuring the diameter of the zone formed around each well.

Identification of potential pigment-producing bacteria

Colony morphology (size, shape, color, margins, opacity, consistency, and elevation) Gram staining and motility characterization of pigment-producing bacteria were done. The biochemical characteristics were found by various biochemical tests such as indole, methyl red, Voges–Proskauer, citrate utilization, urease, oxidase, catalase, and triple sugar iron test. The pure culture of isolate was maintained on nutrient agar slant for further investigation.

Characterization of extracted bacterial pigment

Ultraviolet (UV) spectroscopy analysis of crude pigment

Maximum absorption spectra of the obtained bacterial pigment were performed using UV spectrophotometer. UV spectrum analysis of the obtained bacterial pigment (1 mg/ml) was suspended in a 50 mM Tris-HCl (pH 8.5) solution in the wavelength range between 350 and 750 nm to find out the maximum absorption spectra and Tris-HCl 50 mM (pH 8.5) as blank [9].

Thin-layer chromatography (TLC) analysis

The purified pigment was analyzed by TLC with silica gel. The solvent system consists of chloroform:methanol (9:1; v/v). The chromatography chamber with the solvent was kept for 20 min for the equilibration. The sample was spotted on the silica gel sheet using a capillary tube and air-dried. The TLC sheet was then dipped in the solvent system. After 45 min, the TLC sheet was carefully removed and the retention factor (Rf) value was calculated according to the following equation from the chromatogram [10].

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

Fourier-transform infrared (FT-IR) spectroscopy analysis

The pigment was analyzed using FT-IR spectroscopy. The crude bacterial pigment was encapsulated in KBr at a ratio of 1:100. The IR spectra were collected using a Shimadzu spectrometer within the range of 4000–400 cm^{-1}. The FT-IR spectroscopy is used to analyze the functional group of the extracted pigments.

Minimum inhibitory concentration (MIC) of bacterial pigment

The clinical pathogenic (*E. coli, Klebsiella* sp., *Staphylococcus aureus*, *Pseudomonas* sp., *Proteus* sp., and *Enterococcus* sp.) cultures were inoculated into the nutrient broth and incubated for overnight. The MHA medium was prepared and the Petri plates were sterilized. Then, sterilized medium was poured into the plates and allowed them to solidify. The clinical pathogens were swabbed onto the plates. Well was cut in the agar plates using well puncture. The extracted crude pigment was dissolved in acidified ethanol and 20 µl, 40 µl, 60 µl, 80 µl, and 100 µl were added to the well and incubated these plates at 37°C for 24 h.

RESULTS

Isolation of pigment-producing bacteria

The collected coffee rhizosphere soil samples were subjected to spread plate method on nutrient agar plates. The bacterial cultures were selected from based on the different colony morphology. A total of 30 bacterial strains (YP1–YP30) were isolated from soil sample (Fig. 1). Among the 30 bacterial isolates, four isolates (YP1, YP3, YP5, and YP27) were producing pigments and the pigmented bacterial colonies were used to further screening process.

Screening of antibacterial pigment-producing bacteria

The potential pigment-producing four bacterial isolates were centrifuged and collected; the cell-free supernatants were used to screening as a crude pigment. Among the four pigment-producing isolates, the isolate YP1 showed maximum zone of inhibition against test clinical pathogens (Table 1). The highest activity producing YP1 bacterial strain was used as further analysis.

Identification of the potential pigment-producing bacterial isolate

The bioactive potential pigment-producing bacterial isolate was then preliminary characterized, according to Bergey's Manual of Determinative Bacteriology based on morphological and biochemical characterization. The antibacterial pigment-producing bacterial isolate was translucent, smooth colonies of about 1 mm diameter with profuse brown pigmentations were selected for further studies. The biochemical characterization of the potential bacterial isolate is tabulated in Table 2. The isolated potential bacterial strain was identified as *Roseomonas gilardii* by 16S rRNA sequencing. The isolated antibacterial pigment-producing bacterial isolate YP1 showed 98% similarity with *R. gilardii* and their GenBank accession number is MH324463 (Fig. 2).

Extraction of bacterial pigment

The antibacterial pigments were extracted from YP1 pigment-producing bacterial strain and their extracted pigment (Fig. 3) was characterized for further analysis.

UV–visible spectroscopy analysis of crude pigment

Absorption spectra of pigments from the YP1 strain were studied in the visible range between the wavelength range of 350 and 750 nm and spectrophotometric analysis at the respective wavelength at which maximum absorbance (λ_{max}) was observed at 450 nm (Fig. 4).

TLC analysis of crude pigment

The extracted pigment was separated by TLC with silica-coated TLC plate. The solvent system of chloroform:methanol (9:1) was used for the separation of pigment. TLC showed the presence of a pigment which migrated as brown component in the sheet with Rf 0.82.

FT-IR analysis of crude pigment

The extracted crude pigment was characterized by FT-IR spectrum. Based on the FT-IR spectra, pigment had containing the carboxylic acids, alkenes, phenols, alkanes, and primary amines functional groups (Fig. 5).

Minimal inhibitory concentration of pigment against clinical pathogens

The pigment was tested for antibacterial activity against six selected clinical pathogens such as *S. aureus*, *Klebsiella* sp., *Enterococcus*, *E. coli*, *Pseudomonas* sp., and *Proteus* sp. by well diffusion method. The maximum zone of inhibition observed in *E. coli* bacterial strain (Table 3 and Fig. 6).

DISCUSSION

A total of 30 morphologically different bacterial strains were isolated from the coffee plant rhizosphere soil sample in Yercaud hills region, Tamil Nadu. Among the 30 bacterial strains, only four bacterial strains producing antibacterial pigments. However, the brown pigment-producing colonies were found predominant. Earlier studies in 15 pigment-producing bacteria were isolated from eight soil samples

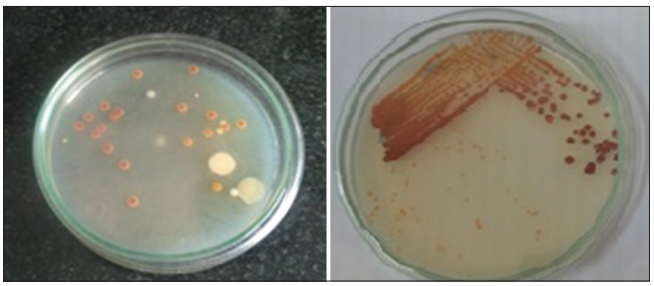

Fig. 1: Isolation of pigment-producing bacteria

Table 1: Antibacterial activity of pigment-producing bacterial isolates

Bacterial crude extract	Zone of inhibition (mm)				
	***Klebsiella* sp.**	***Enterococcus* sp.**	***Pseudomonas* sp.**	***E. coli***	***Proteus* sp.**
YP1	42	41	35	39	38
YP3	21	25	29	20	23
YP5	21	32	29	17	24
YP27	24	-	34	19	21

E. coli: *Escherichia coli*

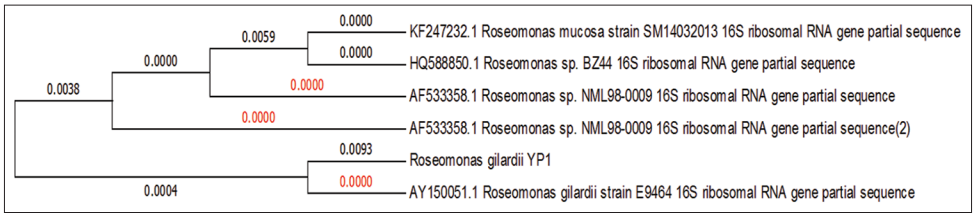

Fig. 2: Phylogenetic analysis of bacterial isolate

Fig. 3: Extraction of potential bacterial pigment

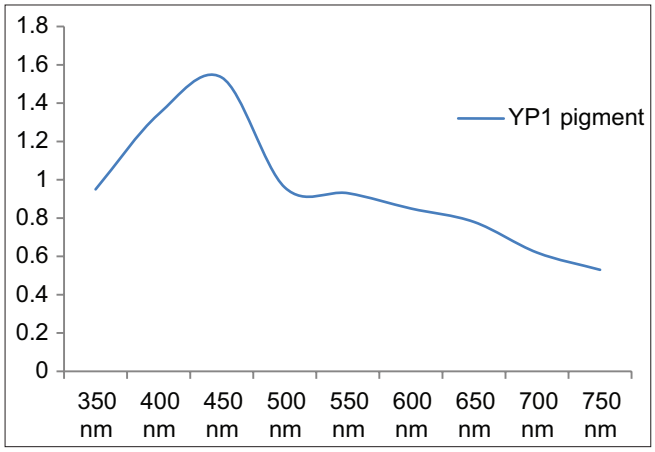

Fig. 4: Ultraviolet spectra absorption of YP1 strain

collected from different places of Dhaka city. The isolated colonies were of the following colors: Red, brown, pink, black, violet, blue, green, cream, golden, dark orange, and light yellow. Among those, the yellow pigment-producing bacterium colonies were most dominant [11,12]. The previous reported that the soil and water samples collected from different parts of Pune were used for the isolation of pigment-producing bacteria. Five pigment-producing bacteria were identified and characterized which were red, yellow, cream, and light orange in color [8]. The present study report that the isolates were purified by quadrant streaking on nutrient agar incubated for 48 h at 37°C. After incubation, pure cultures of each isolates were preserved in 30% glycerol at −20°C. The potential antibacterial pigment producer was then characterized by Bergey's Manual of Determinative Bacteriology based on morphological and biochemical characterization. The molecular identification of the potential bacterial isolate YP1 was identified as *R. gilardii* by 16S rRNA sequencing. In earlier reported that the pigment producers were then identified according to Bergey's Manual of Determinative Bacteriology [13] based on Gram staining, biochemical characteristics, and growth pattern on selective and differential media. The strains were found to belong to the genera *Aeromonas* (20%), *Pseudomonas* (20%), *Chromobacterium* (13.3%), *Flavobacterium* (6.7%), *Bacillus* (13.3%), *Xanthomonas* (6.7%), and *Escherichia* (20%) [11]. A taxonomic identification of the test isolates under study was reported by performing Gram's test, studying cultural characteristics of the isolates on selective and differential media and biochemical tests based on the Bergey's Manual of Determinative Bacteriology. Biochemical tests performed were IMViC, oxidase, and catalase tests. Samyuktha and Sayali Naphade Mahajan were identified that the pigment-producing bacteria were carried out the three isolates which were found to be Gram-negative coccobacilli [14,15]. The intracellular antibacterial pigment was extracted by various methods which were used centrifugation and addition of acidified ethanol so that the cell gets lysed and intracellular pigment can be extracted. The extracted pigment was showed in brown color. Vora *et al.* were extracted the pigment by different solvents with their different concentrations, they are acetone, ethyl acetate, chloroform, and methanol which were used. There was no pigment extraction observed in chloroform and ethyl acetate solvent followed by acetone and methanol are the solvents, which have the capacity to extract the pigment from the bacterial cell. However, the highest extraction of pigment was shown in methanol and the combinations of acetone:methanol (2:1) solvent system showed the maximum extraction (85%) of pigment [10]. In a marine isolate, *Vibrio* sp. pigment was hydrophobic nature so that maximum extraction of the pigment was extracted using different solvents such as ethanol, methanol, chloroform, ethyl acetate, petroleum ether, acetone, and distilled water which also have been considered to find the suitable solvent for effective extraction [16]. The Yercaud region isolates of the four pigments (YP1, YP2, YP5, and YP27) extracted from isolates showing clear zone inhibition against *Klebsiella* sp. (42 mm), *Enterococcus* sp. (41 mm), *Pseudomonas* sp. (35), and *E. coli* (39) for YP1 strain; *Klebsiella* sp. (21 mm), *Enterococcus* sp. (25 mm), *Pseudomonas* (29 mm), and *E. coli* (20 mm) for YP2; *Klebsiella* sp. (21 mm), *Enterococcus* sp. (32 mm), *Pseudomonas* sp. (32 mm), *E. coli* (17 mm), and *Proteus* sp. (24 mm) for YP5 strain; and *Klebsiella* sp.

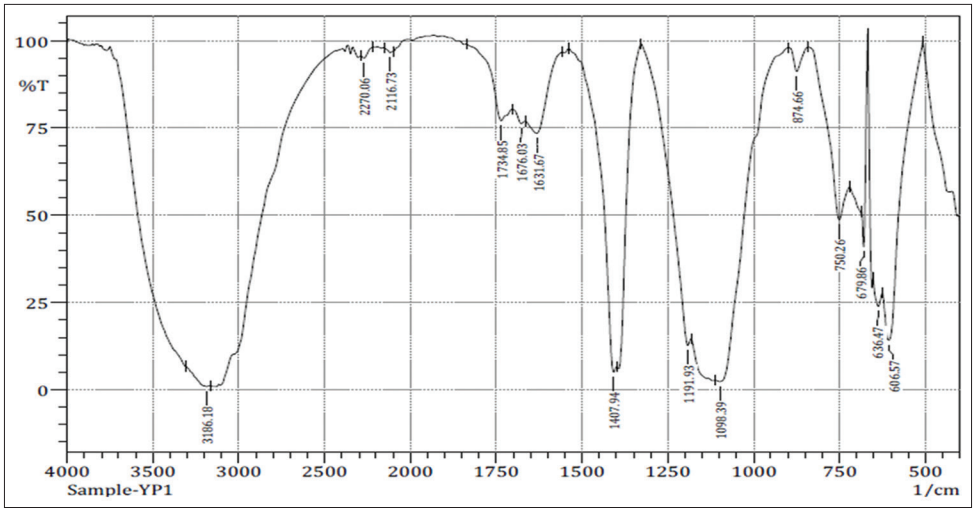

Fig. 5: Fourier transform infrared characterization of pigment

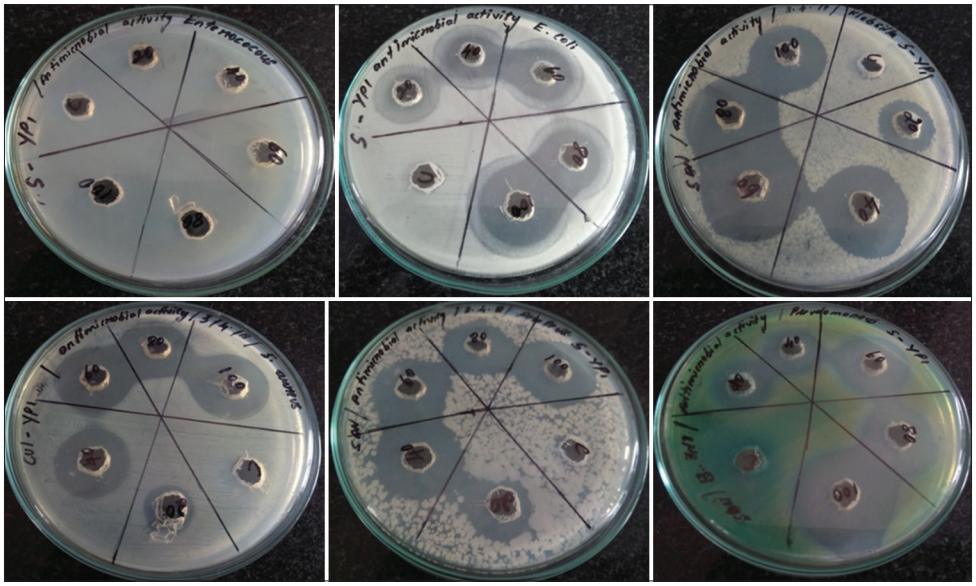

Fig. 6: Minimum inhibitory concentration of YP1 bacterial pigment

Table 2: Biochemical characterization of potential isolate

S. No.	Characterization	Strain YP1
1.	Gram staining	Gram-negative cocci
2.	Catalase test	Positive
3.	Oxidase	Negative
4.	Indole	Positive
5.	Methyl red	Negative
6.	Voges–Proskauer	Negative
7.	Citrate utilization test	Positive
8.	Urease test	Positive
9.	TSI	Negative

Table 3: MIC of bacterial crude pigment

Test pathogen	Concentration of crude pigment (mg/ml)				
	20 µl	40 µl	60 µl	80 µl	100 µl
E. coli	21 mm	26 mm	27 mm	28 mm	30 mm
Pseudomonas sp.	21 mm	21 mm	26 mm	27 mm	28 mm
S. aureus	14 mm	21 mm	23 mm	23 mm	25 mm
Enterococcus sp.	-	-	-	-	-
Klebsiella sp.	16 mm	23 mm	25 mm	25 mm	26 mm
Proteus sp.	16 mm	23 mm	25 mm	25 mm	26 mm

E. coli: *Escherichia coli*, *S. ureus*: *Staphylococcus aureus*. MIC: Minimum inhibitory concentration

(24 mm), *Enterococcus* sp. (resistance), *Pseudomonas* sp. (34 mm), *E. coli* (19 mm), and *Proteus* sp. (21 mm) for YP27; more zone of inhibitory was observed in YP1 strain when compared to other strains for all human pathogen. In earlier reported that the prodigiosin pigment was extracted from isolate showed the clear inhibition zone against *Bacillus cereus* (12 mm), *S. aureus* (7 mm), and *E. coli* (6 mm) [10]. In another report showed the prodigiosin pigment activity against *S. aureus* (17.5 mm), *B. cereus* (10.5 mm), and *E. coli* (resistance), but this report showed that the antibacterial activity of prodigiosin pigment against *B. cereus* was higher than compared with *E. coli* [17]. The UV–visible absorption spectrum of antibacterial pigment from the YP1 strain was studied in the visible range of the wavelength of 350–750 nm and spectrophotometric analysis at the respective wavelength which maximum absorbance was observed in 450 nm. In earlier, *Vibrio* sp. producing pigment was observed in maximum spectrum that was 536 nm in UV-visible spectrometry [16]. Similarly, the prodigiosin pigment from *Serratia* sp. was showed the strong absorbance in the UV region [10,18]. The extracted pigment was separated by TLC with

the solvent system used for chloroform:methanol (9:1). TLC showed the presence of a pigment which migrated as brown component in the sheet with Rf as 0.82. The purified pigment was analyzed by TLC with silica gel G-60. The solvent system consists of chloroform:methanol (95:5; v/v). The chromatography chamber with the solvent was kept for 20 min [10].

CONCLUSION

The polyphenolic rich coffee plant rhizosphere constitutes have extremophilic condition in addition to the lower atmospheric temperature prevailing in Yercaud that greatly influences bio-geo nutrient recycling by retarding. Extremophiles have the tendency to acquire potential characteristics for their survival in competitive environment. Hence, the presence study focused on pigment-producing bacteria from extremophilic condition and found a higher pigment-producing activity with potential antimicrobial characteristic in the isolates. This study thus attempted to explore the extremophilic microbial ecology for pigment production with its antimicrobial characteristics. The identified isolates from soil were found to the YP1 pigment strain, able to produce pigment. In this regard, this study is an initiative approach toward the use of biocolorants which find its applications in numerous sectors as an alternative for synthetic chemicals. To conclude, microbial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications.

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AUTHORS' CONTRIBUTIONS

N. Siddharthan and R. Sandhiya are performed and analyzing this research work; N. Hemalatha is conceived and designing this research work.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS' FUNDING

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