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Research Article

EVALUATION OF CYTOTOXIC ACTIVITY OF ETHYL ACETATE EXTRACT OF PIGMENT FROM *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Objective: Bacterial pigments have promising applications in food, cosmetics, textile, and therapeutics. Pigments from microbial origin are stable, safer, cost effective, easy production, and extraction and thus preferred over other natural sources. Under this backdrop, isolation and characterization of pigment-producing bacteria and analysis of bioactivity of the pigment were the aim of the study. From the literature studies, the pigment production was found to be influenced by various physical factors which directed the study toward optimization of physical parameters for pigment production.

Methods: Isolation of pigment-producing bacteria from water sample, cultural, and microscopic identification was done as per the standard protocol. Extraction of pigment by solvent extraction was carried out and its antibacterial and cytotoxic activity was assayed.

Results: Molecular characterization of the bacteria resembled the query sequence of the isolate to 99% with *Pseudomonas aeruginosa* strain. Extraction of pigment by solvent extraction method resulted in crude pigment extract with antibacterial activity against Gram-negative bacteria (17 mm zone of inhibition) at 100 µg/ml concentration. Pigment showed dose-dependent inhibition on proliferation of HeLa cells at the concentration of 345.83 µg/mL.

Conclusion: From the above results, it was evident that the pigment extracted from the bacterial isolate *Pseudomonas aeruginosa* strain JBT18N was therapeutically potential.

Keywords: Pigment, Antibacterial, Anticancer, MTT Assay.

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INTRODUCTION

Since a decade, there was a robust consumer call for more natural merchandise in therapeutics. Discovery of newer molecule to be used as chemotherapeutic agent has become the need of time [1]. The use of microorganisms for biological reason has end up an effective opportunity to control pathogens [2]. Bioactive compounds because of their safer and extensive applications are preferred over artificial one and thus have augmented inclination closer to substitution of artificial compounds with biological origin. Among natural sources (plants, animals, and insects), microbes are potentially suitable alternatives for the bioactive compounds [3]. Many microbial secondary metabolites are reported with wide range of applications in areas such as food, textile, cosmetics, leather, and pharmaceuticals [4-7]. Numerous plants and microorganisms are known to produce natural colored compounds such as carotenoids, chlorophyll, and anthocyanins [8]. Although the flora produces pigments, it has some drawbacks such as instability toward temperature, light, and non-availability throughout the 12 months. Thus, microbial pigments prove to be advantageous and preferred choice for their applications in various fields [9]. This colorful microbial world has attracted many researchers to explore them for human applications as in textile industries, cosmetics, pharmaceuticals, food industries, and medical [10,11]. Microbial pigments (prodigiosin, astaxanthin, carotenoids, and violacein) are one such metabolites exhibiting multifacet role in medical due to their various biological activities such as antidiabetic, anti-aging, antioxidant, anticancer, and immunosuppressive agents [12]. The interest of researchers toward usage of natural resources for pigments is due to their safe application, simple methods of extraction, cost-effective production, and eco-friendly nature. Many microbial pigments are commercially available as carotenoids, melanin, quinones, violacein, anthraquinone, prodigiosins, chlorophyll, flavins, monascin, etc. [13]. Diverse group of pigment-producing microorganisms is found in natural environment. Thus, natural aquatic bodies serve as diverse bowl of versatile microbes which can be exploited for the production of various bioactive compounds [14-16].

MATERIALS AND METHODS

Bacteriological media from HiMedia Pvt. Ltd. were used for the isolation and mass culture of the isolates. Gram staining kit, solvents, and chemicals for biochemical analysis were used from SRL, E. Merck, India.

Water sample for the study was collected from industrial effluents of Dandeli Paper Mills, Uttar Kannada district of Karnataka [17]. A 500 ml of water was collected in sterile plastic bottles, kept in ice box to restrict the microbial growth. pH of water was checked at the sampling point by pH paper and was transported in controlled conditions to laboratory for further analysis [18,19]. Water sample was serially diluted in saline and plated onto nutrient agar, Mueller-Hinton media, and Zobell Marine Agar, at pH-7, incubated at 37°C for 24 h [20]. All the dilution plates from all media were examined for the pigment-producing bacterial isolates. Pigmented bacterial isolates were pure cultured and one set was preserved in refrigerator for further studies [7]. Mass culture was done with 500 ml nutrient broth in four flasks. The flasks after inoculation were kept for incubation at 37°C for 24 h.

Optimization of parameters

Optimization of fermentation methods is an essential approach needed for high stage production of valuable fermentative products. Medium optimization is one in all the important techniques for purchasing maximum pigment yield and it involves numerous elements which include medium additives, operating conditions, pH, temperature, aeration

and agitation, and so on [21]. The bacterial isolate in the study was also observed for its pigment production at varied physical parameters [22].

The experiment was set up in sets for parameters such as pH, temperature, incubation time, and agitation.

1. The sterile nutrient broth with pH 7, 8, 9, 10, 11, and 12 was inoculated with the bacteria culture and incubated at 37°C.
2. The sterile nutrient broth with pH 7 was inoculated with the bacteria culture and incubated at −4°C, 4°C, 37°C, RT, and 40°C in static condition.
3. The sterile nutrient broth with pH 7, 8, 9, 10, 11, and 12 was inoculated with the bacteria culture and incubated at RT in rotary shaker and in static condition.
4. The sterile nutrient broth with pH 7, 8, 9, 10, 11, and 12 was inoculated with the bacteria culture and incubated at 37°C for 24 h, 48 h, 98 h, and 120 h.

Identification of test organism

The pigmented bacterial colonies from all dilutions were microscopically observed for Gram identification. Further, they were also observed for cultural characters as size, shape, color, elevation, margin, texture, opacity, consistency, motility, and endospore forming. To analyze the isolate at molecular level, 16s rRNA sequencing was carried out. The query sequence was blast to analyze the phylogenetic homology.

Extraction of the crude pigment

The bacterial broth was processed for the extraction of pigment by liquid-liquid extraction method using ethanol, methanol, and ethyl acetate solvents. Ethyl acetate was chosen as a solvent for extraction of the pigment due to its solubility in it. The incubated bacterial broth was centrifuged at 8000 rpm for 10 min. The colored supernatant was collected in a fresh tube and the cell pellet was discarded. The supernatant was treated with equal volume of ethyl acetate and the extract was collected by separating funnel and dried at room temperature [22].

Antibacterial activity of pigment

Pigment was assayed for its antibacterial activity against four human bacterial pathogens as follows:

1. *Staphylococcus aureus* – ATCC 25923/MCC 2408
2. *Bacillus subtilis* – ATCC 6633/MCC 2048
3. *Pseudomonas aeruginosa* – ATCC 27853/MCC 2080
4. *Escherichia coli* – ATCC 11775/MCC 2079 (T)

Petri plates containing 20 ml nutrient agar were seeded using cotton swab with 24 h (old) culture of bacterial strains. Wells were cut (8 mm diameter) and 50 µl of different concentrations of test samples (100, 200, and 400 µg/ml) and standard ciprofloxacin (100 µg/ml) was added. The plates were then incubated at 37°C for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well [23].

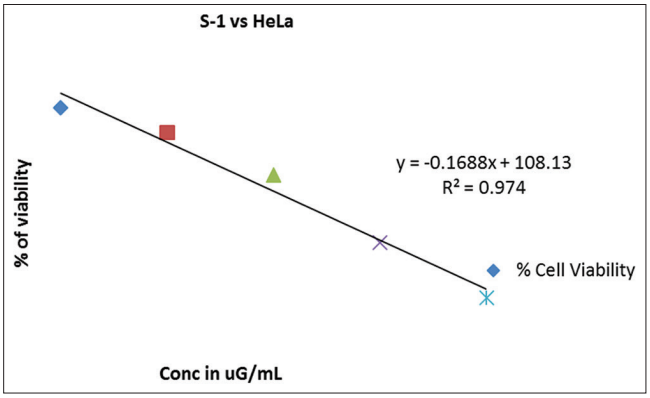
MTT cell proliferating assay

A 200 µl cell suspension was seeded in a 96-well plate at required cell density (10,000 cells per well), without the test agent. The cells were allowed to grow for about 24 h. Appropriate concentrations of the test agent were added. The plates were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. MTT reagent of 0.5 mg/mL concentration was added to the plates, wrapped with aluminum foil to avoid exposure to light and incubated for further 3 h. The MTT reagent was replaced by 100 µl of solubilization solution (DMSO). The absorbance was read on ELISA reader at 570 nm and 630 nm used as reference wavelength. The IC_{50} value was determined using linear regression equation, i.e.,,Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph [24].

RESULTS

Isolation and identification of pigment-producing bacteria

Out of all the bacterial colonies produced on all the media used for isolation, prominent blue-green color producing bacterial isolate was selected for the study. Colony characteristics were studied with microscopic observation of Gram staining and structural staining. The isolate was Gram-negative, non-spore-forming, and non-capsulated motile rods (Table 1). Results of biochemical tests revealed that the isolate was negative to indole, methyl red, and Voges–Proskauer, urease, and coagulase test and positive to citrate catalase and oxidase nitrate reduction test (Table 1).



Graph 1: Percentage of cell viability with concentration

Table 1: Morphology and biochemical tests of bacterial isolate

Colony morphology		Biochemical analysis	
Characteristics	**Results**	**Tests**	**Results**
Shape	Rods	Indole	−ve
Motility	Motile	MR	−ve
Capsule	Non-capsulated	VP	−ve
Endospore	Non-sporing	Citrate	+ve
Flagella	Single flagella	Catalase	+ve
gram staining	Gram negative	Oxidase	+ve
Pigment	Positive (+ve)	Urease	−ve
		Nitrate reduction	+ve
		Coagulase	−ve

Table 2: Effect of different physical parameters on growth and pigment production by *Pseudomonas aeruginosa*. '+'no growth and no pigment production, '+'minimal growth with light pigmentation, '++' moderate growth with good pigmentation, '+++' luxuriant growth with intense pigment production

Parameter	pH of media					
	7	8	9	10	11	12
Growth	++	+	++	+	+	+
Pigmentation	+++	++	+++	++	+++	+
	Incubation temperature (°C)					
	-4°C	4°C	RT	37°C	40°C	
Growth	-	+	++	++	-	
Pigmentation	-	+	++	++	-	
	Incubation time (Hrs)					
	24	48	72	96	120	
Growth	+	+	+	+	+	
Pigmentation	+	++	+++	+	+	
	Agitation					
	Rotary shaker			**Static condition**		
Growth	+			+		
Pigmentation	+++			++		

Optimization of growth conditions for pigment production

Out of all the media (nutrient broth, Mueller-Hinton media, and Zobell Marine broth) used in the study consistent and intense pigment production was observed in nutrient broth. *Pseudomonas aeruginosa* showed purple color pigment production within 24 h at room temperature on rotary shaker at pH-8 and green color at 37°C in rotary shaker at pH-8. Further, the range of colors was observed at different pH from brown, purple, green, and blue at pH −7, 8, 9, 10, 11, and 12 (Fig 1), (Table 2).

Molecular characterization

Identification of the bacterial isolate under study was done by 16s rRNA sequencing at NCCS, Pune. The BLAST result of the query sequence revealed the isolate to be 99% similar to *Pseudomonas aeruginosa* (Fig 2).

1 ctacacatgc aagtcgagcg gatgaaggga gcttgctcct ggattcagcg gcggacgggt
61 gagtaatgcc taggaatctg cctggtagtg ggggataacg tccggaaacg ggcgctaata
121 ccgcatacgt cctgagggag aaagtggggg atcttcggac ctcacgctat cagatgagcc
181 taggtcggat tagctagttg gtggggtaaa ggcctaccaa ggcgacgatc cgtaactggt
241 ctgagaggat gatcagtcac actggaactg agacacggtc cagactccta cgggaggcag
301 cagtggggaa tattggacaa tgggcgaaag cctgatccag ccatgccgcg tgtgtgaaga
361 aggtcttcgg attgtaaagc actttaagtt gggaggaagg gcagtaagtt aataccttgc
421 tgttttgacg ttaccaacag aataagcacc ggctaacttc gtgccagcag ccgcggtaat
481 acgaagggtg caagcgttaa tcggaattac tgggcgtaaa gcgcgcgtag gtggttcagc
541 aagttggatg tgaaatcccc gggctcaacc tgggaactgc atccaaaact actgagctag
601 agtacggtag agggtggtgg aatttcctgt gtagcggtga aatgcgtaga tataggaagg
661 aacaccagtg gcgaaggcga ccacctggac tgatactgac actgaggtgc gaaagcgtgg
721 ggagcaaaca ggattagata ccctggtagt ccacgccgta aacgatgtcg actagccgtt
781 gggatccttg agatcttagt ggcgcagcta acgcgataag tcgaccgcct ggggagtacg
841 gccgcaaggt taaaactcaa atgaattgac gggggcccgc acaagcggtg gagcatgtgg
901 tttaattcga agcaacgcga agaaccttac ctggccttga catgctgaga actttccaga
961 gatggattgg tgccttcggg aactcagaca caggtgctgc atggctgtcg tcagctcgtg
1021 tcgtgagatg ttgggttaag tcccgtaacg agcgcaaccc ttgtccttag ttaccagcac
1081 ctcgggtggg cactctaagg agactgccgg tgacaaaccg gaggaaggtg gggatgacgt
1141 caagtcatca tggcccttac ggccagggct acacacgtgc tacaatggtc ggtacaaagg
1201 gttgccaagc cgcgaggtgg agctaatccc ataaaaccga tcgtagtccg gatcgcagtc
1261 tgcaactcga ctgcgtgaag tcggaatcgc tagtaatcgt gaatcagaat gtcacggtga
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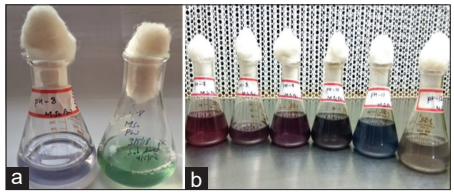

Fig. 1: (a) Violet and light green coloured pigment production by *Pseudomonas aeruginosa* in culture broth on rotary shaker at room temperature and 37oC respectively, (b) Range of coloured pigments in culture broth produced by *Pseudomonas aeruginosa* at pH -7, 8, 9, 10, 11,and 12

Antibacterial activity of crude pigment

Crude pigment extracts in ethyl acetate from the bacterial isolate *Pseudomonas aeruginosa* JBT18N were tested for its antibacterial activity against four human bacterial pathogens. A 17 mm zone of inhibition was observed only against *Escherichia coli – ATCC 11775/MCC 2079 (T)* at 100 µg/ml concentration with positive control of 100 µg/ml standard ciprofloxacin antibiotic (Fig 3), (Table 3).

Anticancer activity of crude pigment extract

MTT assay of crude pigment extract from the bacterial isolate *Pseudomonas aeruginosa* JBT18N was tested on HeLa cells with IC_{50} concentrations at 345.83 µg/mL (Graph 1).

DISCUSSION

Cancer chemoprevention employs natural or synthetic compounds to prevent, arrest, or cease carcinogenesis [25]. The use of microbial metabolites as a remedy of most cancers has been utilized since long time [26]. Since the past 10 years, compounds derived from bacteria were tested for their antibacterial and anticancer activity [27]. This includes the use of naturally occurring microbial biomass, bacterial strains to be engineered for the expression of desired genes. Advanced and novel approach includes the use of bacteria or bacterial metabolites as proteins, enzymes, and pigments that can cause inhibition of growth or the apoptosis of tumor cells [28]. In this regard, natural pigments can be extracted from plants, insects, animals, and microbes (fungi, bacteria, and actinomycetes). Among all the bacterial pigments are preferred over the other natural sources for their easy isolation, cost-effective substrates, purification techniques, diverse range of pigment production, and safer applications [29].

In the present study, the pigment production was affected by many physical factors such as temperature, pH, light, agitation, and incubation time. Intense and consistent pigment production was recorded at 37°C, pH 10.8, and incubation for 72 h with agitation. The optimum growth of bacterial isolate TM and production of carotenoid at 37°C with pH 7.2 after 144 h of incubation with agitation in a media supplemented with 1% yeast extract, 0.75% casamino acids, 25% NaCl, 4% MgSO4, and

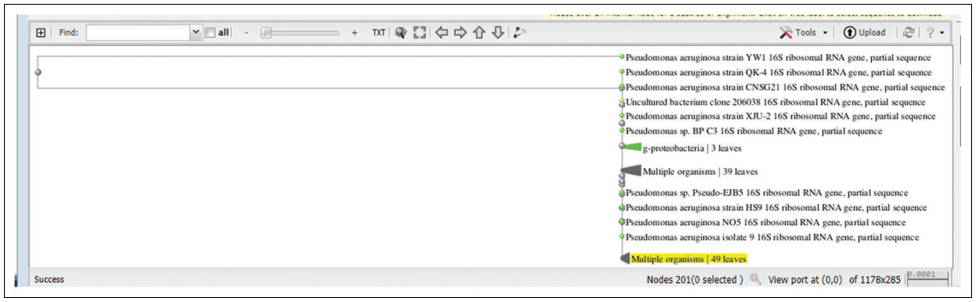

Fig. 2: Phylogenetic tree of *Pseudomonas aeruginosa* JBT18N

Table 3: Minimum inhibitory concentration in µg/ml of pigment extract on bacterial pathogens-1. *Staphylococcus aureus*, 2. *Bacillus subtilis*, 3. *Pseudomonas aeruginosa*, 4. *E. coli*

S. No.	Bacterial strain	Std. – ciprofloxacin 100 µg/ml	100 µg/ml	200 µg/ml	400µg/ml	800µg/ml
1.	*Staphylococcus aureus – ATCC 25923/MCC 2408*	39 mm	-	-	-	-
2.	*Bacillus subtilis – ATCC 6633/ MCC 2048*	39 mm	-	-	-	-
3.	*Pseudomonas aeruginosa – ATCC 27853/MCC 2080*	39 mm	-	-	-	-
4.	*Escherichia coli – ATCC 11775/MCC 2079 (T)*	40 mm	17 mm	-	-	-

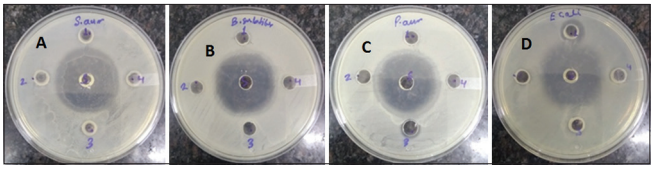

Fig. 3: Antibacterial activity of crude pigment extract from *Pseudomonas aeruginosa* JBT18N on A. *Staphylococcus aureus*, B. *Bacillus subtilis*, C. *Pseudomonas aeruginosa*, D. *Escherichia. coli*

0.2% KCl [30]. Optimum pigment production was also found at pH-5, 27°C in rotary shaker by Patil *et al.* [31].

The crude pigment extract was active against *Escherichia coli – ATCC 11775/MCC 2079 (T)* at 100 g/ml con. with 17 mm inhibition by Kirby-Bauer method, which can be compared with the similar studies carried out with pigment prodigiosin against *Staphylococcus aureus, Bacillus cereus, Candida albicans, C. parapsilosis,* and *Cryptococcus* sp. [32].

To evaluate the pigment for its cytotoxic activity against the most emerging cancer in women across the world human cervical cancer, the crude pigment extracted from the bacterial isolate in the study was assayed for its potency on HeLa cell lines and was found to be cytotoxic. A novel red pigment extracted from marine *Arthrobacter* sp. G20 showed moderate anticancer effects on an esophageal cancer cell line, KYSE30 [13].

CONCLUSION

Microbes serve as a choice of source for the natural therapy for various diseases. From ancient times, microbial metabolites and their derivatives have proved to be an excellent, reliable therapeutic value. Marine microbes due to their unique and versatile adaptability provide an opportunity to researchers for the discovery newer potential drugs. The present study focuses on the characterization of pigment-producing bacteria with potential bioactive compound. Many biological active compounds are extracted from microbes so far as antibiotics, antibacterial, antifungal, anticancer, antidiabetic, and antioxidant, immunosuppressive, and so on. Microbial metabolites are so diverse in their applications that still there is lot of scope of exploring newer microbe and unique metabolite as potential drug.

CONFLICTS OF INTEREST

Authors do not have any conflicts of interest related to the manuscript.

AUTHORS' CONTRIBUTIONS

Principal author: Performed the collection of sample, designing of experiment, isolation, extraction process, and wrote the manuscript. Corresponding author supervised the work at every step and helped in evaluation of manuscript. The coauthor supported by providing infrastructure facilities and equipment to carry out the work.

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