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Research Article

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Isolation, characterization and application of violacein pigment from *Chromobacterium violaceum*

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Abstract

Chromobacterium violaceum is a gram-negative, facultative anaerobic, non-spore-forming bacterium that produces violacein, an indole derivative pigment. Scope of the study includes isolation, characterization & application of the isolated pigment from the bacterium. A water sample was collected from a pond near SNGIST Arts & Science College. In this study, organism's development was assessed in nutrient agar & liquid pineapple waste supplemented with 1% tryptophan. Production of the pigment was higher in nutrient agar supplemented with 1% tryptophan. Violacein pigment was extracted with ethanol & ethyl acetate. Extracted pigment was characterized using a UV-Vis spectrophotometer, which showed a maximum absorbance peak at 540nm. Presumptive test for pigment indicated that it was colourless at acidic pH & turned green at alkaline pH. Solubility test confirmed violacein is insoluble in oils. Pigment displayed antimicrobial activity against *Pseudomonas* sp., with a zone of inhibition measuring 20mm and antioxidant activity, showing higher scavenging activity compared to the standard ascorbic acid. Viability for use as a colourant in textiles & candle making was established, necessitating further research into its qualities.

Keywords: Violacein, Characterization, Stability, Agricultural waste material, Application

Introduction

Pigments are intensely coloured substances that are produced either by living organisms or by chemical processes, essential for metabolism & development. They are found in plants, animals & microorganisms. They are present in vegetables, fruits & flowers; are also found in skin, eyes, & in other animal structures. Among the pigment producers, microorganisms are the dominant one. Common pigments include carotenoids, melanins, quinones, flavins, prodigiosins & monascins, violacein or indigo. This study mainly focuses on Violacein, a purple coloured pigment produced by *Chromobacterium violaceum*. *C. violaceum* is a gram negative facultative anaerobic, non-acid fast

small rods, or non-sporing-coccobacillus from the *Neisseriacea family*. They have a growth range from 15-40°C. Optimal growth is achieved at 30-35°C. Violacein is a naturally occurring bis-indole pigment with antibiotic properties & also produced by other bacterial strains like *Collimonas*, *Duganella*, *Janthinobacterium*, *Microbulbifer* species & *Pseudoalteromonas*.

Materials and Methods

Sample collection: A water sample collected from a pond nearby the college was cultured in minimal agar medium to isolate pigmented bacteria. Sample collected was plated on nutrient agar medium supplemented with 1% Tryptophan (concentration 1mg/1ml) with optimum conditions like pH 6 & temperature 30°C. After incubation, violet coloured colonies formed & were subcultured onto nutrient agar. Production of crude violacein was studied by two methods: (i) Strains were grown in a 100ml nutrient broth in a conical flask with 1% tryptophan added to one of the flasks as a precursor for enhanced growth & violacein production (Yoshitoshi et al., 2003). Optimum temperature of 30°C & pH of 6 was provided. (ii) A series of 500ml Erlenmeyer flasks, one containing a mixture of 50ml liquid pineapple waste & nutrient agar & the other containing a mixture of 50 ml liquid pineapple waste, nutrient agar & 1% tryptophan was prepared. Slants of the medium were inoculated & incubated overnight at 30°C. Pigment production occurred only in tryptophan supplemented tubes (Ahmad et al., 2012).

Purification of cultures and maintenance: Pigmented bacterial isolate was purified on nutrient agar plates with 1% Tryptophan & incubated at 30°C for 24hrs. Isolates were subcultured & stored in refrigerator. Organism was streaked on a nutrient agar plate, incubated at room temperature for 24hrs & analyzed for colony characteristics, gram staining, motility & biochemical tests. Pigment production by organism was higher on nutrient agar supplemented with 1% Tryptophan & on liquid pineapple waste medium with 1% Tryptophan. Nutrient agar with 1% Tryptophan was selected as production medium of this study.

Extraction of the pigment: Violacein was extracted from broth using ethyl acetate & ethanol by centrifugation. 100ml of broth was centrifuged at 8000rpm for 10mins. Supernatant was collected & discarded. Cell pellet was rinsed with deionized water & centrifuged again to recover cells by discarding supernatant & centrifuged till there was no residual pigment in cell pellet after extraction. Same procedure was done in extraction of pigment by ethyl acetate. Intensity of colour of pigment was more in extraction using ethanol than ethyl acetate. Ethanol was selected for extraction of pigment in further studies.

Presumptive test for extracted pigment: Culture broth was centrifuged using different solvents such as ethanol & ethyl acetate. After removal of pellets, supernatant was tested against acidic & alkaline conditions. **S**tability of pigment in different oils like coconut oil & olive oil was observed & colour changes in pigment were noted (Nakata et al., 1979).

UV - Visible absorption of extract: Ethanolic extract as well as ethyl acetate extract was determined for maximum wavelength λ_{max}, by using UV-Vis spectrophotometer (Shimadzu UV-1601PC) between 400 & 700 nm. Maximum band was observed. Ethanol & ethyl acetate was used as blank.

Thin layer chromatography analysis of pigment: To detect the compound, 100ml of samples were subjected to chromatography on TLC silica gel plates using solvents like isopropanol/ ammonia/water (8: 1: 1). Samples were allowed to run for about ¾th of the plate & observed under UV light[4]. R_f (retention factor) values of the spot was calculated using the formula:

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

Antioxidant assay by DPPH: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by mixing 0.5ml of DPPH solution (0.002% in methanol) with 0.5ml of different concentrations of each fraction & standard (ascorbic acid) in separate tubes (Kekuda et al., 2010). After 30mins of incubation in dark room temperature, optical density was measured at 517nm using UV spectrophotometer. Absorbance of DPPH control (without extract/ standard) was noted. Scavenging activity was calculated using the formula: Scavenging activity = [(A – B)/A] × 100 where, A is absorbance of DPPH control and B is absorbance of DPPH in the presence of extract.

Evaluation of antimicrobial activity of violacein: Test pathogens (*Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp., & *Staphylococcus* sp.*)* were obtained from SNGIST ASC laboratory & subcultured on minimal agar & stored at 4°C. Antimicrobial activity was assessed using agar well diffusion method (Duran et al., 1983). Four wells of 5mm in diameter were made on Mueller Hinton agar plate using a sterile cork borer. Ethyl acetate extract of violacein (100 µl & 150 µl) was added to the wells. Positive (Amoxicillin) & negative controls (ethyl acetate) were also added. After 24hrs of incubation at 37°C, zones of inhibition were measured & tabulated.

Application of pigment:

Violacein as a dye on cotton cloth material:

Pigment in ethanol was used as the stock solution. Equal-sized 5cm^2 cotton cloth pieces were dyed with 200ml ethanol-based pigment solution in a warm surface & dried for 24hrs & dipped in thiourea solution for 30mins at 50°C with white cloth material as the control. Dyed cloth was washed with soap solution for 30mins & with tap water & dried. Absorbance of soap solution after washing was measured at 535nm in a UV-Visible spectrophotometer with an appropriate blank (G. Krishna et al., 2008).

Violacein as a candle: Wax melted in a double boiler for 10-15 mins with stirring frequently & ethanol extract of violacein was mixed in. A wick, dipped in wax, was attached to the bottom of the container i.e., an egg shell. Let the wax sit for 5mins to harden & melted wax was poured into shell & left to set at room temperature.

Results

Enhanced growth was observed on nutrient agar plate 1% tryptophan incubated under room temperature. In nutrient broth with 1% tryptophan, *C. violaceum* showed characteristic purple pellicle formation. Growth occurred only in tube having liquid pineapple waste medium (with 1% Tryptophan). Pure culture plates displayed violet, low convex, smooth, & non-gelatinous colonies. Microscopy revealed long, rod-shaped, Gram negative cells in clusters. Motility of organism was confirmed at the edge of the drop. Biochemical test results are summarized in Table 1. Production media selected was nutrient broth with 1% Tryptophan due to enhanced growth at pH 6 & temperature of 30°C. Pigment was extracted from broth culture using ethanol & ethyl acetate by centrifugation; ethanol having better colour than ethyl acetate.

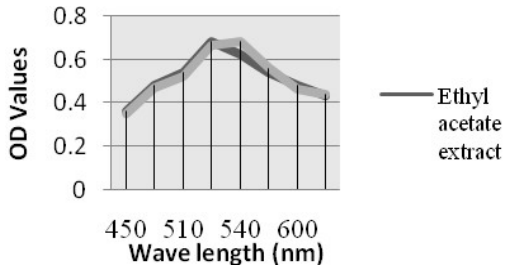
Table.1: Result of the biochemical tests

Indole	-
Methyl red	-
Voges-proskauer	-
Citrate utilization	+
Triple sugar iron agar	K/A
Mannitol motility	+
Urea hydrolysis	-
Catalase	+
Oxidase	+

(+): positive & (-): negative

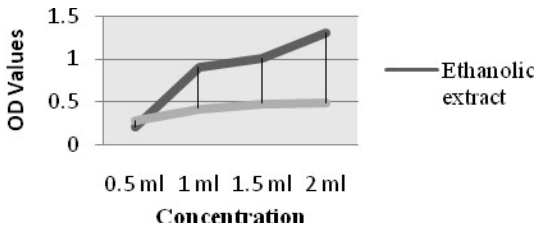
Violacein presence was confirmed by positive presumptive test; it turned colourless at high pH (10.5) & green at low pH (2). It changed from dark purple to light violet colour in oils like olive & coconut oil, but remained insoluble due to polarity & charges of pigment molecules & solvent properties. Maximum absorbance was recorded at 520nm for ethyl acetate extract and 540nm for ethanolic extract, scanned across a wavelength region of 400-700nm using a UV-Vis spectrophotometer.

Graph.1: Representation of UV-Visible absorption of the extract.



Pigment was subjected to TLC yielding an R_f value of 0.7. Distance travelled by pigment violacein was found to be 4.2cm & by solvent front was 5.3cm. DPPH radical scavenging activity of extract was compared with ascorbic acid; it showed high scavenging activity than ascorbic acid, with 50% activity at 1 mg/ml concentration. The extract showed strong antibacterial activity against *Pseudomonas* sp. with a zone of inhibition of 20mm compared to other organisms showing zone of 7mm. Wash performance tests confirmed the pigment's retention on cloth after washing, indicating potential as a dye in future. A candle made with violacein was successful with its antibacterial & antioxidant properties conferring biological safety (fig: 1&2).

Graph 2: Antioxidant assay of the extract



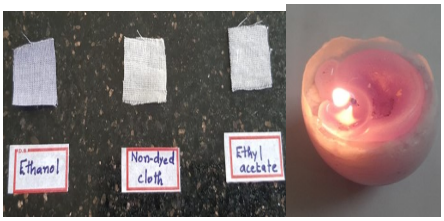


Figure: 1) violacein as dye for cloth & 2) as a candle

Discussion

The bacterium from water body was identified as *C. Violaceum* based on test results matching previous studies (Yoshitoshi et al., 2003). It grew optimally at 30°C & pH 6 in nutrient broth, with low convex, violet, smooth & non-gelatinous colonies as described by Richards (1993). Identification was confirmed through gram staining, pigmentation & biochemical tests. Its strong growth on liquid pineapple waste with 1% Tryptophan is attributed to the agricultural waste's high levels of sugars, organic acids, protein, phenolic compounds, & several trace elements which are essential for bacterial growth (Tanaka et al., 1999). Rapid propagation time of this bacterium suggests the potential of agricultural waste for commercial-scale production. Spectrophotometric analysis showed that extracted pigment in ethanol & ethyl acetate had adsorption peaks at 540nm & 520nm respectively, due to chromophore groups, like alkene & carbonyl present in the pigment. Alkene groups contribute to the conjugation effect. Both ascorbic acid & violacein exhibited enhanced scavenging activities with increasing concentration, with violacein demonstrating significant radical scavenging activity. DPPH radical contains an odd electron, responsible for strong absorbance at 517nm; it is a deep purple colour (Mohan et al., 2007). Violacein showed maximum antibacterial activity against Gram positive bacteria, particularly against *Pseudomonas* sp. (20mm of zone of inhibition), while showing less activity against *E.coli*, *Klebsiella* sp., *Staphylococcus* sp. Pigment is suitable for use as a natural dye to impart violet colour to textiles. Candle prepared from this pigment has antibacterial & antioxidant properties making it biologically safe. Future studies could explore the use of violacein in other fields like leather industry & beauty products such as hair colour, eyeshadows, & lotions after the cytotoxicity studies in mammalian cells to ensure safety.

Conclusion

Bacteria produce pigments for various purposes & have a vital role on Earth. In this study, violacein extracted from *C. violaceum* was found to be a suitable biopigment for cloth dye & candles. The easy extraction of Violacein from bacterial cultures for commercial interest & easy maintenance support the use of bacteria over plant sources. This makes it a promising candidate for various clinical, cosmetic & environmental applications.

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