Extraction and Characterization of the Pigment Violacein from
Chromobacterium violaceum and Its Antibacterial Properties

Evangelin Ezhil Malar S., Krishnaveni N.*, Achuth Jayakrishnan and Ronald

Department of Microbiology. PSG College of Arts & Science, Coimbatore. Tamil Nadu, India
*Corresponding Author: krishnaveni.narayanaswamy@gmail.com

ABSTRACT

Chromobacterium violaceum, is a saprophyte found in soil and water. Though normally considered non–pathogenic to humans, they can be opportunistic pathogens with great virulence. A non–diffusible violet pigment is produced by this Gram negative bacterium. The biosynthesis and biological activities of violacein and the diverse effects of this pigment have been studied at a wider level. C.violaceum also produces other antibiotics such as aerocyanidin and aerovacin, which exhibit activity against Gram positive and Gram negative bacteria. The present study focuses on characterization of this bacteria and extraction of the pigment Violacein with ethanol and purification by filtration. The Violacein pigment was purified using Thin Layer & Column Chromatography. GC-MS analysis of the extracted violacein has been performed. The purified pigment may be incorporated as a Food additive or a natural food colouring agent.

Key words: C.violaceum, Violacein, Natural Pigment, Toxicity study.

INTRODUCTION

Violacein, a purple pigment produced by Chromobacterium violaceum was reported[1, 2] based on the visible absorption spectra and is now found in Bergey’s Manual of Systematic Bacteriology[3]. C. violaceum is a saprophytic bacterium that is generally considered to be nonpathogenic for humans. It has been reported from soil and water habitats of tropical and subtropical areas of several continents.

Chromobacterium violaceum is a species of bacteria that are Gram-negative, non-acid fast small rods or cocobacilli and non-spoore formers. They are 0.6-0.9μm x 1.5-3.0μm in size and exhibit bipolar staining. Motility of C. violaceum is achieved by means of a single polar flagellum and up to four antigenically and structurally distinct lateral flagellae.

C. violaceum are facultative anaerobes with a growth range from 15-40°C. Optimal growth is achieved at 30-35°C. They characteristically produce violet colonies on nutrient agar and usually grow on MacConkey agar. Sterile rice grains and reduced strength nutrient agar have been successfully used to isolate them from soil or water. Colonies are low convex, violet, smooth and not gelatinous[4].

Purple pigment producing bacteria generally belong to the group of Chromobacterium, Iodobacteria and the Janthinobacterium genera. Violacein the major pigment produced by these bacteria has various antagonistic properties. The Violacein is a bactericide[5, 6], a trypanocide[7, 8], a tumoricide[9] and in addition it also exhibits anti-viral activity[10].

Chromobacterium violaceum could be used for the production of Violacein, a purple coloured pigment, which has antibiotic characteristics particularly against soil amoebae and trypanosomes. In addition to all these characteristics, C. violaceum are opportunistic pathogens and occasionally cause serious pyogenic or septicemic infections of mammals, including man and cause fatal septicemia starting from skin lesions to many liver and lung abscesses. Strains of virulent Chromobacterial endotoxin, is more reactive than avirulent ones. Virulent strains are able to survive attack from phagocytic cells by elevated levels of superoxide dismutase and catalase. Infections due to soil or water contamination with the organisms can be
quite varied, ranging from mild diarrhea to septicemia leading to a rapid death. Liver and lung abscesses have been reported and similar infections in animals have also been reported. The study was focused on pigment extraction from *Chromobacterium violaceum* and molecular analysis of the pigment compounds.

**MATERIALS AND METHODS**

**Growth and maintenance of *Chromobacterium violaceum***

*Chromobacterium violaceum* standard strain (MTCC. No.8071) was obtained from MTCC Chandigarh, India. The strain was cultivated on nutrient agar and blood agar and the colonies grown were taken for further interpretation for their microscopic, cultural and biochemical characterization. Gram staining and cultural characteristics were performed.

**Determination of Growth Curve of *Chromobacterium violaceum***

50 ml of nutrient broth was prepared and sterilized and culture was inoculated. Absorbance was measured at 0th time point at the wavelength of 660 nm. Nutrient broth that was not inoculated was used as blank and the optical density was measured in spectrophotometer. Values were recorded. In order to obtain a good growth curve, measurements of optical density was observed for every 2 hours up to 48 hrs.

**Pigment extraction**

Culture was inoculated in broth and was incubated for 3, 5 and 7 days. The sample was centrifuged to remove the white cell pellet. The supernatant contained the total extractable pigments. To this about 25ml of 95% Methanol was added and centrifuged at 7500 rpm.

**Purification and Characterization of Violacein Pigment**

**Thin layer Chromatography**

For the detection of compound, 100 l of samples were subjected to chromatography on TLC silica gel plates. The solvents used were isopropanol/ammonia/water (8: 1: 1). The samples were allowed to run for about ¾th of the plate. Plates were viewed under UV light and the Rf values of the spot was calculated using the formula:

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

**GC-MS analysis**

A GC system (Trace GC Ultra, Thermo, USA) linked to a (single quadrupole mass selective detector) DSQII (Thermo, USA) and controlled by the software Xcaliber™ (Version 1.4 SR1, Thermo electron Co. Inc. USA) equipped with an auto-sampler (AS 3000, Thermo, USA) and a DB-5MS MSD capillary column, 30 m × 0.25 mm ID, 0.25 im was used for characterization of the compounds. Helium (99.999%) was used as the carrier gas, at the flow rate of 0.8 ml min⁻¹. The oven temperature of GC was increased from 150°C (3 min. hold) to 275°C at a rate of 15°C min⁻¹. The injector was kept at 200 °C, the transfer line at 280 °C, and the ion source at 230 °C. The ionization energy was 70 eV and the mass spectrometer was run at SIM (single ion monitoring) mode at m/z 143 (Cataldi et al., 2007). The peaks obtained in the GC-MS results were analysed to understand the components of the purple pigment violacein.

**FTIR Spectrophotometer**

The UATR-FTIR spectra of the pigment violacein were recorded using a Bruker Tensor (Germany) instrument in the range of 450-4500 cm⁻¹ with a resolution of 4 cm⁻¹. The absorption spectra of the pigment were recorded and analysed.

**Antagonistic activity of Violacein against pathogens**

A pure culture of *Escherichia coli*, *Klebsiella* sps., *Proteus* sps., *Pseudomonas* sps, *Salmonella* sps, *Serratia* sps, *Bacillus* sps, *Staphylococcus* sps. and the fungal species *Trichoderma* sps., *Penicillium* sps., *Fusarium* sps. and *Aspergillus niger* were cultivated in suitable broth for testing its sensitivity pattern for the pigment. Mueller-Hinton agar was prepared and samples
were swabbed and excess inoculum was removed. The prepared pigment containing discs were dispensed onto the surface of the previously inoculated agar plate in which lawn of test bacterial culture has been made. The plates were placed in an incubator set to 37°C within 15 minutes after the discs were applied.

After 16 to 18 hours of incubation, each plate has to be examined for uniform zone of clearance and confluent lawn of growth which is possible, if the plates were satisfactorily streaked, and the inoculum were correct. The diameter of the zones of inhibition has to be measured, including the diameter of the disc [13,16,17].

RESULTS AND DISCUSSION

Chromobacterium violaceum standard strain (MTCCNo.8071) obtained as a lyophilized culture, was revived at suitable conditions. The nutrient broth which was inoculated with the standard culture showed characteristic growth of Chromobacterium violaceum with purple pellicle formation. The purple colour formation was observed from bottom to the surface of the broth.

Chromobacterium violaceum colonies grown were observed for characteristic colony morphology (Fig.1) and have been used for all further analysis. The pure mother culture was maintained in nutrient agar and broth.

The stained smear was observed under microscope and the bacterial cells were observed to be long, rod shaped gram negative bacteria which were found in clusters.

C. violaceum purple pellicle formation

Purple colonies on Nutrient Agar plates

Fig. 1

Hanging drop method for motility analysis revealed an active serpentine motility. Various biochemical tests were performed. These results obtained were compared with standard Bergey’s classification to compare the characteristics of the bacterium. The colony characteristics of C. violaceum observed in Nutrient agar and Blood agar plates as dark purple, small, circular, raised and easily emulsifiable colonies were observed on the nutrient agar plates and

Fig. 2 : Growth curve of Chromobacterium violaceum

Typical α- Haemolytic purple colonies were observed on blood agar plate.

The morphology of cells were observed to be long, rod shaped gram negative bacteria which were found in clusters. Hanging drop method revealed an active serpentine motility. IMViC results were suitable to be identified as the Chromobacterium and glucose was the only sugar fermented.

Fig. 3 : Violacein Pigment production observed and Extraction of violacein pigment
The growth cycle was measured for about 0 to 30 hours at 560 nm. It was observed that the growth of the bacterium increased exponentially up to 14 hours after which a stationary phase occurred followed by a decline in growth indicating the generation time approximately to be between 10 to 14 hours.

Dark violet coloured pigment was obtained. The absorbance value of the samples was measured using spectrophotometer. The pigment was subjected to Thin layer chromatography and the Rf value of the sample was analyzed with the standard values. The distance travelled by the pigment violacein was found to be 4cms and by the solvent was found to be 6 cm, the Rf value thus calculated was 0.6.

The optical density of the pigment was measured (Fig. 4) for 3rd, 5th and 7th day respectively, the value obtained on the 7th day of incubation of culture was found to be higher and proves that larger amount of pigment can be extracted with increased growth period.

The pigment was subjected to Thin layer chromatography and the Rf value of the sample was analyzed with the standard values the retention factor was calculated using the following formula:

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

The distance travelled by the pigment violacein was found to be 4cm and by the solvent was found to be 6cm and the Rf value thus calculated was 0.6.

GC-MS analysis was carried out for the pigment violacein extracted using methanolic solvent in order to check for the various compounds present. Various peaks were obtained which was studied for its characteristic compounds. The mass spectrum (Figure 6) shows the molecular ion m/z. 343; the spectroscopic studies conducted earlier during extraction of the pigment shows strong absorption at the visible region due to resonance of violacein; and the spectrum (Figure 6) presents the stretching bands at 21.456 and 25.313 representing Hexadecanoic acid and 9-Octadecanoic acid due to C-O bonds.

The ATR-FTIR spectra were recorded (Fig. 5) using a Bruker Tensor instrument in the range of 450-4500 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\). The chemical changes on the pigment extracted 5th day were analyzed using ATR-FTIR spectra. This has been compared with the FTIR spectrum of another pigment sample extracted on the 7th day to identify any addition or deletion or increase in intensity of any compounds. The samples collected on two different days did not show much of difference, which represents that the pigments have
common functional such as aliphatic amines, aldehydes and phenolic compounds as represented in IR chart which are distributed in them right from the initial period of production.

**Antibacterial Efficacy studies of Violacein**

Antibacterial activity for the pigments extracted on 3\textsuperscript{rd}, 5\textsuperscript{th} and 7\textsuperscript{th} day of production was checked against various microorganisms such as *E. coli*, *Pseudomonas*, *Klebsiella*, *Salmonella* sp., *Staphylococcus aureus*, and *Bacillus* sp. The pigments showed considerable antibacterial activity against *Staphylococcus* and *Salmonella* having zone of inhibition up to 15mm. The pigments showed considerable antibacterial activity against *Staphylococcus* and *Salmonella* having zone of inhibition up to 15mm.

Antibacterial activity of the pigment against other bacteria did not show promising results. An antibacterial effect of the pigment has given way for promising applications of this pigment as natural colourants in textile, food additives and other applications.

**CONCLUSION**

The Violacein pigment from *Chromobacterium violaceum* is a characteristic rare natural colourant, thus can be used as alternative source of purple dye/pigment in various applications. The pigment has to be verified for its toxicity effects for application as food colourant.

**REFERENCES**

