Original Article

Utilization of Affordable Natural Substrates for Violacein Production by *Chromobacterium violaceum*

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Abstract

Beside expanding awareness among the population for natural dyes over the synthetic dyes for environment friendly factors, there is demand for dyes and pigments from natural resources. Natural dyes and pigments can be obtained from various sources like, plants, insects, plant root, tree bark and microorganism. Bacterial pigment (violacein) produced by *Chromobacteriumviolaceum*, is highly in demand due to its pharmacological properties. In this study, initially two soil samples were collected from MCRC, Taramani, Chennai. *C.violaceum* was isolated from soil sample by serial dilution method. Screenings for the effective growth of *C. violaceum* in natural substrate medium (of low-cost) were performed. *C.violaceum* producesdarkvioletpigment called "VIOLACEIN". As*C.violaceum* is a slow growing organism, the more days it is kept for incubation the more pigment is produced. Effective natural substrate medium was chosen for growth study. Appropriate solvent system for efficient extraction of violet pigment (violacein) was screened. The pigment is highly soluble in polar organic solvents and insoluble in water and non-polar organic solvents. In this study, ethyl alcohol was screened as suitable solvent for pigment extraction from the natural substrates. It was then employed for further application (Figure 1).

Keywords: *Chromobacteriumviolaceum*, violacein, natural substrate, low-cost medium, ethyl alcohol.

Introduction

The creation and application of natural pigments as substitutes for artificial colorants has drawn increasing attention from throughout the world in recent years. Despite being widely used in sectors like textiles, food, cosmetics, and medicines, synthetic dyes have drawn criticism for their possible negative effects on human health and the environment. As a result, there is a greater need for natural pigments, which are biodegradable and frequently have bioactive qualities[1].

Natural pigments are derived from various sources, including plants and microorganisms[2, 3, 4]. Plant-based pigments, such as carotenoids, anthocyanins, and betalains, are commonly extracted from fruits, vegetables, roots, seeds, and tree bark[5]. These pigments not only impart color but also offer health benefits, including antioxidant, anti-inflammatory, and anticancer properties[4].

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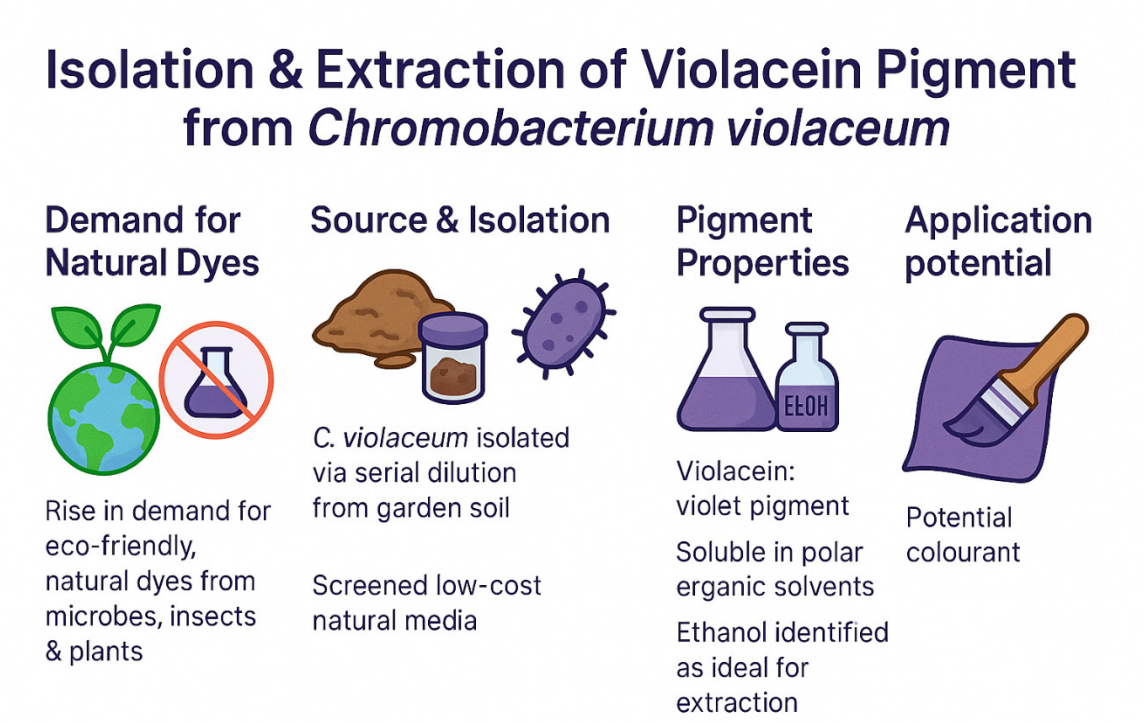

Figure 1: Graphical abstract, representing the research flow from isolation of pigmented bacteria to its application Created using ChatGPT.

Microbial pigments have garnered significant attention due to their advantages over plant-derived pigments. Microorganisms like bacteria and fungi can produce a wide range of pigments, such as carotenoids, melanins, and violacein, through fermentation processes[3, 6]. Additionally, microbial production is not affected by seasonal variations and can utilize low-cost substrates, including agro-industrial wastes, enhancing sustainability and cost-effectiveness[3].

The exploration of natural pigments, particularly those produced by microorganisms, presents a promising avenue for developing eco-friendly and health-conscious alternatives to synthetic dyes. This study focuses on the isolation of *C. violaceum*, a bacterium known for producing the violet pigment violacein, and the identification of cost-effective natural media to optimize pigment production.

C. violaceum is a Gram-negative, facultative anaerobic coccobacillus belonging to the Neisseriaceae family. It produces violacein, a violet indole-derived pigment with notable antimicrobial, anticancer, and antioxidant properties. Recent studies have highlighted violacein's potential in treating colorectal cancer and inflammatory gastric lesions[7].

However, the high production cost of violacein remains a challenge. To enhance the economic viability of violacein production, research has explored the use of low-cost substrates. To address this, recent research has focused on utilizing low-cost substrates, such as agro-industrial wastes, to enhance the economic viability of violacein production.

Experimental Procedures

Starting with isolation, identification and pigment production of violacein-producing Bacteria (MV1) related studies took about 13-15 days. This study was carried out from Dec 2018- Jun 2019.

Sample Collection and Isolation of Bacteria

Soilsamples were collected from different location at Shri A.M.M.MurugappaChettiarResearch

Centre, Taramani, Chennai, for isolating violet colour pigment producing bacteria. Soil samples were processed by serially diluting under aseptic conditions byfollowing the standard methods [8].The bacteria were originally isolated by plating the dilutions on the nutrient agar plate and incubated at room temperature for 24 hours to 48 hours. The pigmented colonies were picked and sub-cultured periodically on the nutrient agar.

Morphological, Biochemical and Molecular (16S Rrna) Studies Foridentification of Bacteria

The morphological and biochemical characteristics of pigmented bacterialMV1 was carried out by following the standard methods[8]. Biochemical analysis andcarbohydrate fermentation pattern were also performed by using a HiAssortedBiochemical Test kit (HiMedia) and HiCarbo kit (HiMedia). Pigmented bacterial isolates were cultured on nutrient agarmedium, sealed with paraffin wax and submitted to the Gujarat State BiotechnologyMission (GSBTM), Gujarat for conducting 16S rRNA sequence analysis.

Growth and Production of Pigment (Violacein)Bymv1 using Low Cost Substrates

The production profile of crude violacein was studied by growing the pigmented strains on around 15 different naturalmediadescribed in Table 1, incubated for 30±2 °C up to 72 h. At the end of 72 hours of incubation period, the growth medium was taken for pigment extraction by using ethyl alcohol. The preliminary analysis of the natural growth medium showed significant growth and pigment production in medium amended with pumpkin seeds followed by peanut oil cake. Henceforth, these two natural substrates were considered for further studies to record growth (for every 24h upto144h).

Characterization of Violet Pigment

Violet pigment (violacein) from *C. violaceum* was extracted. Pigment was purified by thin layer chromatography method. TLC was performed on a glass plate (20 x 10cm) with Silica Gel G. the slurry was prepared with double distilled water (1:2), coated on a pre cleaned glass plate, air dried for an hour at room temperature and activated by placing it in hot air oven for 60mins at 100°C [9]. Violet pigment which was recovered with ethanol was spotted on the activated TLC plate. The solvent system which was used for separation was n-hexane: ethyl acetate (40:60v/v). The TLC chamber was preloaded with solvent and left for saturation for about an hour. Pigment spotted TLC plate was placed inside the tank and observed for the separation and process was stopped when the solvent front reached 80 percent of the chromatogram. The Retention factor (R_f) values were noted and was calculated.

Monitoring of Pigment Production by MV1 Over Time on Specific Growth Media

Growth and pigment production was observed on different natural substrates medium (refer Table 1) and substrates were screened for maximum pigment production. Further, selected substrates (pumpkin seed powder and peanut oil cake powder)were employed separately for monitoring of pigment production over time. In test tube, 1 gram of pumpkin seed powder was taken along with distilled water (10 mL), sterilized and inoculated with loopful of inoculum (MV1). Growth and pigmentation of MV1 was recorded for every 24 hrs upto144 hrs. Similarly, in a test tube, 1 gram of peanut oil cake powder was taken along with distilled water (10 mL), sterilized and inoculated with loopful of inoculum (MV1). Growth and pigmentation of MV1 was recorded for every 24 hrs upto144 hrs.

Pigmentextraction

Culture MV1, inoculated in coconut coir+pumpkin seed powder and peanut oil cake powder were observed for every 24hrs, and culture was centrifuged at 8000rpm for 20mins, and 5mL of 70% ethyl alcohol was added to the pellet and incubated overnight. On the next day, it was centrifuged again to collect pigmented supernatant, extracted pigment was determined by measuring its absorbance maxima (λ_{max}) using UV-Vis spectrophotometer (200nm – 800nm).

Table 1. Composition of Growth Medium (Low cost Substrates):

S. No	Medium	Ingredients Quantity		Inoculum (MV1)
		Substrate (g)	Distilled water (mL)	
1.	Peanut oil cake	20	50	3mL
2.	Sesame oil cake	20	50	3mL
3.	Coconut oil cake	20	10	3mL
4.	Castor oil cake	20	20	3mL
5.	Fresh sugarcane bagasse (Pulverized)	20	20	5mL
6.	Fresh sugarcane bagasse (Pulverized) + Rancid peanut	20	15	5mL
7.	Fresh sugarcane bagasse (Pulverized) + Peanut oil cake	5+10	15	5mL
8.	Dried Sugarcane Bagasse	3	10	5mL
9.	Fresh sugarcane bagasse (Pulverized) + Pumpkin seeds	5+5	15	5mL
10.	Fresh sugarcane bagasse (Pulverized) + Rancid peanut + Mango kernel po wder	5+2.5+2.5	15	5mL
11.	Fresh sugarcane Bagasse (Pulverized) + Moringa seeds	5+5	15	5mL
12.	Fresh Sugarcane bagasse (Pulverized) + Rancid peanut + Azolla	5+2.5+2.5	15	5mL
13.	Coconut coir (Pulverized) + Moringa seeds	5+5	15	5mL
14.	Coconut coir (Pulverized) + Rancid pumpkin seeds	5+5	15	5mL
15.	Spent potato + Dextrose	10+0.4	200	6mL

***Footnote**: All media formulations were prepared in triplicate, and each experimental setup was inoculated and incubated under identical conditions to ensure reproducibility and statistical validity of the observations.*

Applications

Colouring of Candle Wax and Handmade Paper with Violacein as Colourants

Candle wax was purchased from the local market and brought to the laboratory. Wax (20 g) was melted in a container with microwave oven (make: LG) for 10 min. Melted wax was poured in the respective moulds greased with fragrance oil and left undisturbed until the wax was set. On the other hand, unused single side papers were used for making handmade papers and coloured.

Textile Dyeing and Oil Painting

Ready for dyeing cotton fabric (200 TC) was procured from Vaibhav processing mills Pvt Ltd, Erode. Mordants employed before dyeing with violet pigment from *C. violaceium* MV1were, 1. Alum, 2. Biosilica, 3. Tannic acid and 4. Lemon juice. Fabric was treated with 5 % of mordant to wof.

Results and Discussion

Morphological, Biochemical and Molecular (16S Rrna) Studies Of *C. Violaceum*mv1

The morphological characteristics of the MV1 (violet pigment producing) recorded motile and gram negative. Recorded characteristics were also cross referred by following Bergey's Manual of Systematic Bacteriology. The recorded results revealed that the isolate MV1 belonged to genus of Chromobacterium. Violet to purple pigment, smooth, convex, and circular with entire edges were recorded on Nutrient agar (NA) plates. Bacterial isolate MV1 was submitted to the Gujarat State Biotechnology Mission (GSBTM), Gujarat for genetic identification (16S rRNA) and MV1 recorded 99 % identity with *C.violaceum*(Accession no. KY672928). Individual culture colony was streaked on nutrient agar (NA) slants for further use (Figure 2: Isolated *C.violaceum*MV1 from the soil sample).

Figure 2: *C. violaceum* MV1 on Pumpkin seed medium

Growth and Production of Pigment (violacein) by *C. violaceum*MV1

Growth of *C.violaceum*MV1 was observed in 15 different natural substrates medium. And out of 15 different combinations of natural substrate sources, (i) Peanut oil cake, (ii) Fresh Sugarcane Bagasse + Pumpkin seeds and (iii) Coconut coir + pumpkin seeds, observed to produce pigment. Effective extraction of violacein was confirmed by recording the absorbance at 576 nm (Table 2; Figure 3).As mentioned by author in 2015 [11] microorganisms are the most adaptable tools in biotechnology to produce a variety of molecules including enzymes, antibiotics, organic acids and pigments and hence more intense efforts are required to have a cheap organic substrate for the growth of colour producing microorganisms. However, as stated in an article published in 2017 [12], during the last few decades, environmental concerns related to the utilisation of the synthetic dyes, have enthused R&D scholars all over the countries to explore new ecofriendly substitutes for minimising their negative environmental impacts and various aspects of bio colourant applications. One should also concentrate on the parameters that influence

the microbes in producing pigment.Violet pigment (violacein) from *C violaceum* MV1 was also purified using TLC. Pigment was eluted into a single violet band with R_f value 0.53 cm was recorded, which was in agreement with results documented in 2012.[10].

Table 2. Absorbance recorded by pigment produced in different Natural Substrates

S. No.	Substrate	Growth at end of 72hrs	Extraction of pigment from substrates (overnight soaking in)	Solvent extraction (centrifugation)	Peak observed at (200-800)	Absorbance (nm)
1.	Peanut oil cake	Enormous growth	0.5grams in 5mL of ethyl alcohol	6000rpm,10mins	576	**0.493**
2.	Sesame oil cake	Adequate growth	0.5grams in 5mL of ethyl alcohol	6000rpm,10mins	575	0.226
3.	Coconut oil cake	No growth	-	-	-	-
4.	Castor oil cake	No growth	-	-	-	-
5.	Fresh Sugarcane Bagasse	Requirement of additional nitrogen supply	-	-	-	-
6.	Fresh sugarcane Bagasse + Fresh Peanut	Adequate growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	576	0.252
7.	Fresh sugarcane Bagasse + Peanut oil cake	Spotted at very few places	-	-	-	-
8.	Dried Sugarcane Bagasse	No growth	-	-	-	-
9.	Fresh Sugarcane Bagasse + Pumpkin seeds	Enormous growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	577	**0. 486**
10.	Fresh Sugarcane bagasse + Fresh peanut + Mango kernel powder	No growth	-	-	-	-
11.	Fresh Sugarcane Bagasse + Moringa seeds	No growth	-	-	-	-

Continue....

12.	Fresh Sugarcane bagasse + Fresh peanut + Azolla	Adequate growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	539	0.274
13.	Coconut coir + Moringa seeds	Adequate growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	575	0.099
14.	Coconut coir + pumpkin seeds	Enormous growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	577	0.489
15.	Spent potato + Dextrose	Adequate growth	0.5grams in 5mL of ethyl alcohol	8000rpm, 20mins	578	0.221

***Footnote**: All experiments were carried out in triplicates. Values reported for absorbance represent the mean of three independent observations. Variations across replicates were within acceptable experimental limits.*

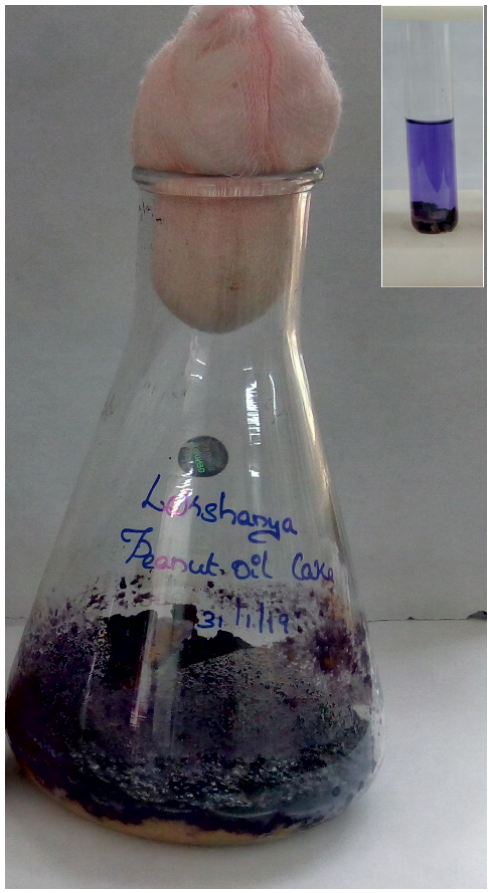

Figure 3: *C. violaceum* MV1 on peanut oil cake medium

Monitoring of Pigment Production by *C. violaceum* MV1 over time on Specific growth Media

Growth and pigment production by *C. violaceum* MV1 on Pumpkin seed medium (Fig. 3-a) was maximum on 5th day (120 h) and peanut oil cake medium (Fig. 3-b) also recorded for maximum pigment production on 5th day (120 h) (Table. 3). The violacein production can also be quantified using a spectrophotometer, after an extraction and purification process at 576 nm [10, 14]. Due to the increased conc. of pigment, it was diluted in the ratio of 1:5 (Pigment: 70% ethyl alcohol). Pigment from the biomass was extracted using soxhlet apparatus for about 6 cycles, with ethanol. The pigment obtained was evaporated using a rotary evaporator and concentrated pigment was transferred in a bottle, until further use. The manipulation of environment and nutrition has been shown to have substantial impacts on the quantity and variety of secondary metabolite production[15, 16, 17, 18, 19, 20, 21, 22]and optimisation of the growth medium may have insightful effects on the output and steps involved with natural product synthesis[23]. It is very vital to investigate on the influence of growth medium in the production of secondary metabolites in microbes [24].

Table 3. Pigment production by *Chromobacterium violaceum* MV1 on natural substrates

S. No.	Incubation Period (Hours)	Coconut Coir + Pumpkin Seeds (OD_{576} ± SD)	Peanut Oil Cake (OD_{576} ± SD)
1	24	0.411 ± 0.015	0.319 ± 0.005
2	48	0.432 ± 0.012	0.406 ± 0.012
3	72	0.523 ± 0.013	0.520 ± 0.005
4	96	0.747 ± 0.010	0.687 ± 0.010
5	120	0.963 ± 0.056	0.850 ± 0.007
6	144	0.342 ± 0.019	0.529 ± 0.027

***Footnote**: Each value represents the mean of three independent replicates ± standard deviation (SD). Optical density was measured at 576 nm to monitor pigment productionacross different incubation periods.*

Applications

Wax and Paper Colouring

One millilitre of violacein pigment was added to the wax mould with melted wax, blended well and allowed set. Violacein did not get mixed with wax, and it simply dispersed evenly and appeared as tiny violet dots in wax. Ten percent of pigment was used for colouring the paper pulp. Violacein could bind well with the cellulosic fibres of pulp and coloured the paper in grey hue (dark grey–RGB Colour code: 180, 174, 178). Colouring of white coloured handmade paper was also performed. Handmade white paper was cut in (60 mm × 15 mm) circular shape. Each paper was poured with 10 mL of violacein and air dried, violacein dyed paper in grey hue (silver sand–RGB Colour code: 194, 193, 191). Developed colour shades for colouring paper were in accordance with the research carried out in 2012 [26, 13].

Textile Dye and Oil Paint

Mordanted fabrics (5% to w.o.f.) were dyed with violet pigment from *C. violaceium*MV1 and dyed samples were submitted for evaluation at Regional Laboratory, Textile committee (Ministry of Textiles, Govt. of India) Chennai. Violacein pigment dyed fabric eeffectively (Figure 4). Also, violacein pigment was formulated into paint by mixing it with linseed oil and talc powder and was used as an oil paint to paint in canvas board. Developed dye shades in fabric were in accordance with the shades developed in 2016[27].

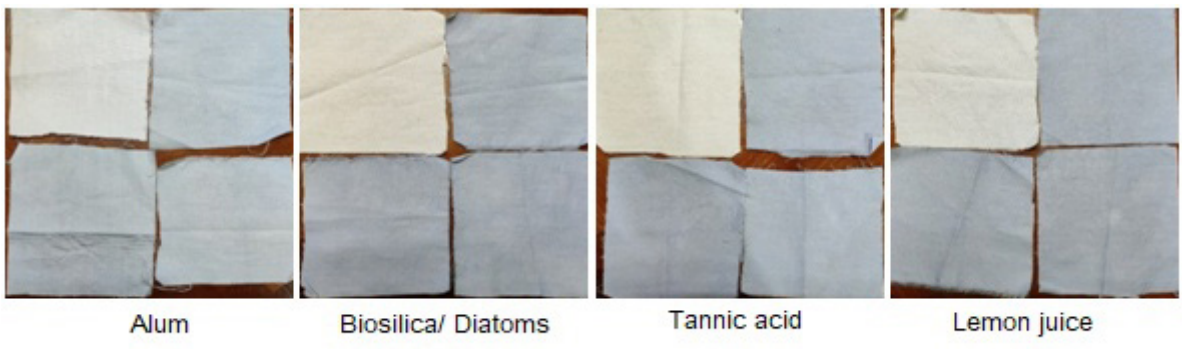

Figure 4: Violacein dyed fabric

Conclusion

Microorganism can serve as a potent natural dye source. Bacterial pigment (violacein) produced by *Chromobacteriumviolaceum*MV1, is highly in demand due to its pharmacological properties.The present work aims to determine the production of *C.violaceum*by using low cost natural substrate medium. Growth of *C. violaceum*is determined / analysed by using different low cost substrates. Based upon maximum pigment production further study was carried out in Pumpkin seeds and Peanut oil cake was chosen for time based production study that recorded enormous production of violacein pigment. The pigment was further used in various applications like oil painting, textile dyeing etc..

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Conflict of Interest: There is no conflict of interest declared.

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