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Molecular identification and optimized production of violacein by *Chromobacterium vaccinii* Strain NFML 5214 isolated from Arunachal Pradesh, India

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ABSTRACT

This study aimed to screen and isolate a purple-violet pigment-producing bacterium from soil samples collected from Talley Valley Wildlife Sanctuary, India, and develop an optimized procedure for enhanced pigment production for potential industrial, agricultural, and medicinal applications. A preliminary study was also conducted to evaluate textile-dyeing properties of the pigment produced by the bacterium. The purple–violet-colored bacterium was isolated on a nutrient agar plate, and a pure culture was prepared and maintained for further studies. The bacterium's identity was established using both morphological and molecular (16S rRNA gene sequence analysis) approaches. An optimum culture medium was developed for enhanced pigment production by adjusting various growth factors, such as pH, temperature, incubation period, and concentration of media components, for maximum pigment production. The isolated bacterium strain NFML 5214 (PV613353.1) was identified as *Chromobacterium vaccinii* based on 16S rRNA gene sequence analysis, which showed 99.07% similarity to *C. vaccinii* strain 21-1 (CP017707.1). The bacterium produced the maximum amount of pigment on the 6th day of incubation at pH 6 and 30°C with optimized concentrations of peptone (7 g/L), beef extract (4 g/L), and NaCl (2 g/L). The total yield of pigments in the optimized medium (192 mg/L) showed a 69.42% increase compared with the yield of the basal medium (113 mg/L). A hot water extract dyeing process of white muslin cloth revealed dark purple fastness. Since *C. vaccinii* is reported to produce violacein and deoxy-violacein, further studies are needed to characterize the pigments and evaluate their potential medicinal properties, such as antimicrobial, antidiabetic, and antioxidant properties, and industrial value, such as pharmaceutical and textile dyes as an alternative to synthetic dyes.

1. INTRODUCTION

Synthetic and natural dyes are used in textile, cosmetic, food processing, paper, and pharmaceutical industries [1,2]. Synthetic dyes are widely preferred over natural dyes due to their low cost and readily availability. There have been growing concerns regarding the continuous and excessive use of synthetic dyes due to their associated health risks, such as allergic reactions, neurocognitive effects, and environmental pollutions [3]. However, natural biological dyes are considered eco-friendly, non-toxic, and non-carcinogenic, largely due to their biodegradable nature [4]. Pigments from living organisms, such as plants, animals, and microorganisms, have been considered as promising alternatives to synthetic dyes. Bacteria and fungi are potential sources of natural pigments because mass production can be achieved at any time through different fermentation techniques [5]. Bacteria can be used as an important source of natural pigment because it is easier to modify them genetically for enhanced pigment production [6]. Numerous exploratory studies have been reported on pigment production by different species of bacteria. Several pigment-producing bacteria have been studied for their potential applications in the food, dyeing, textile, and pharmaceutical industries, including *Bacillus cereus*, *Chromobacterium violaceum, Duganella* species, species of *Micrococcus* and *Salinococcus*, *Serratia marcescens,* and *Yarrowia lipolytica* [7-13].

Violacein is a violet-colored natural pigmented compound synthesized by several bacterial species using tryptophan as a precursor molecule. This pigment compound has been reported to exhibit a broad range of biological activities, such as antibacterial, antiplasmodial, antifungal, antipyretic, antitumor, antiparasitic, antiviral, and ulcer protective properties, and is capable of inducing apoptosis in certain cancer cells [14-21]. The potential of violacein as a bio-dye has also been reported [7]. In addition to their pigment-producing ability, certain

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strains of *Chromobacterium vaccinii* have been reported to show bio-control properties against diseases spread by insects such as moths and mosquitoes [22]. Violacein is a naturally occurring compound reported to be produced by several bacterial species, including *C. vaccinii, C. violaceum, Janthinobacterium violaceum, Duganella violaceinigra, Y. lipolytica,* some species of *Pseudoalteromonas, Iodobacter,* and *Massilia* [23,24]. Most *Chromobacterium* species are isolated from soil and aquatic environments [25]. *C. vaccinii* is a psychrophilic bacterium capable of producing violacein pigment which can thrive and survive in extremely cold environments with soil temperatures ranging between 15°C and 37°C and pH levels between 5.0 and 9.0 [26,27]. The biosynthesis pathway of violacein involves tryptophan as a precursor and can be engineered for large-scale production by regulating the associated cluster of genes and operons [28]. Many species of *Chromobacterium* are categorized as bio-safety level 2 organisms because of their potential to induce opportunistic infections in humans [29]. Although these infections are not highly virulent, they can lead to serious complications, such as sepsis, abscess development, and organ failure, particularly in individuals with compromised immune systems.

The Talley Valley Wildlife Sanctuary (TVWS) in the Lower Subansiri District is located 130 km from Itanagar, the capital city of Arunachal Pradesh, India. TVWS is spread over an area of 337 km^2 and is surrounded by the Pange and Sipu Rivers on its eastern and southern sides, respectively. The primary vegetation comprises temperate broadleaved forests, temperate conifers, and subtropical broadleaved forests. This sanctuary possesses undisturbed pristine climax vegetation comprising subtropical and alpine forests with endangered flora and fauna. Some studies on faunal and floral diversity have been reported over the past two decades [30,31]. However, no study on the bioprospecting of soil microbial diversity with reference to bacteria has been reported from this natural habitat. Therefore, the present study aimed to screen, isolate, identify, and optimize the natural pigment-producing bacterium, *C. vaccinii* from the natural forest soils of the Tale Valley Wildlife Sanctuary.

2. MATERIALS AND METHODS

2.1. Study Site Description and Soil Sample Collection

Soil samples were collected from the Tale Valley Wildlife Sanctuary (TVWS), Arunachal Pradesh, India [Figure 1]. Soil sampling was performed in winter at an average temperature of 1–2°C. Samples were collected from the Tale camp at 27°2.082′ N, 093°57.131′ E, at an elevation of 2369 m above sea level. A total of 27 soil samples were collected, with nine samples from each of three replicate plots (100 m^2). A pre-sterilized polypropylene bag was used for soil collection. The samples were transported to the laboratory, and the nine subsamples from each plot were homogenized to obtain only three replicates. Moisture content and pH were recorded immediately, and the rest of the samples were used for bacterial isolation.

2.2. Isolation of Pigment-producing Bacteria

The stock soil suspension for bacterial isolation was done by dissolving 10 g of the soil sample in 90 mL of sterile distilled water in a 150 mL conical flask. This solution was kept in a shaker at 150 rpm overnight at room temperature. Serial dilution was prepared to obtain a dilution up to 10^{-4} [32]. Screening was performed by inoculating 100 µL of soil suspension (10^{-4}) on nutrient agar medium (NAM) and actinomycetes isolation agar (AIA) plates using the spread plate method. Plates were incubated at 28 ± 2°C for 48 h. Bacterial colonies with natural pigments (deep blue to violet) were isolated from the mixed colonies on nutrient agar plates and subcultured on a fresh NAM plate using the streak plate method [33]. Pure slant cultures were also prepared in test tubes (18 mm × 120 mm, Borosil, India) and stored in the refrigerator at 4°C for further studies [34].

2.3. Morphological Identification

The morphological characteristics of the bacterial colonies, such as form, elevation, margin, texture, and color, were recorded as per the standard procedure of Cappuccino [32]. The Gram staining procedure was followed to classify Gram-positive and negative pigment-producing bacteria [35].

2.4. Bacterial Genomic DNA Isolation and Polymerase Chain Reaction (PCR) Amplification

For molecular identification, a pure bacterial culture was grown in nutrient broth medium (NBM), and genomic DNA was extracted from a 24-h-old culture using the modified CTAB method [36]. Genomic DNA was amplified using a pair of 16s rRNA primers, B27F (5'AGAGTTTGATCCTGGCTC3') and U1492R (5'GGTTACCTTGTTACGACTT3'). The PCR reaction was performed in a volume of 25 µL, consisting of 2.5 µL 10× buffer, 0.5 µL dNTPs, 1 µL each of forward and reverse primers, 1 µL Taq polymerase (1U), 1 µL bacterial genomic DNA, and 18 µL sterile water. The PCR program was run in a thermal cycler (T100, BIO-RAD) with an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C (45 s), primer annealing at 58°C (45 s), and extension at 72°C (45 s), final stabilization at 72°C for 5 min, and storage at 4°C. The DNA product was visualized using 1% agarose gel electrophoresis. The PCR product was purified, and Sanger sequencing was performed in duplicate for both forward and reverse reactions at Eurofins Genomics (India) Pvt. Ltd., Bangalore, India.

The DNA sequences were assembled and annotated using Geneious Prime (Dotmatics) and submitted to GenBank (NCBI). A sequence homology search was performed using nucleotide BLAST (NCBI). The evolutionary phylogenetic tree was constructed using MEGA12 software [33].

2.5. Optimization of Violacein Pigment Production in Liquid Culture Medium

The maximum pigment production was evaluated by optimizing the concentrations of media compositions and key physical parameters such as temperature, medium pH, and bacterial broth culture incubation period. The effect of media composition was evaluated by changing the concentrations of individual components in the NBM [37]. The optimum concentration of each composition with the maximum pigment production was selected and used for subsequent experiments throughout the study. Following each optimization step, the OD of the broth cultures and controls (uninoculated medium) was measured at 570 nm. All experiments were performed in triplicate.

2.5.1. Effect of temperature and pH on the production of violacein

The temperature optimization was performed by culturing C. *vaccinii* in standard nutrient broth media at various temperatures (i.e., 20°C, 25°C and 30°C) for 7 days [10]. The effect of hydrogen ion concentration (pH) on pigment production was assessed by culturing the bacterium in nutrient broth containing different pH levels ranging from 5.0 to 9.0 using a modified protocol [38]. The pH of the media was adjusted using 1 N HCl and 1 N NaOH. The pigment production under each culture

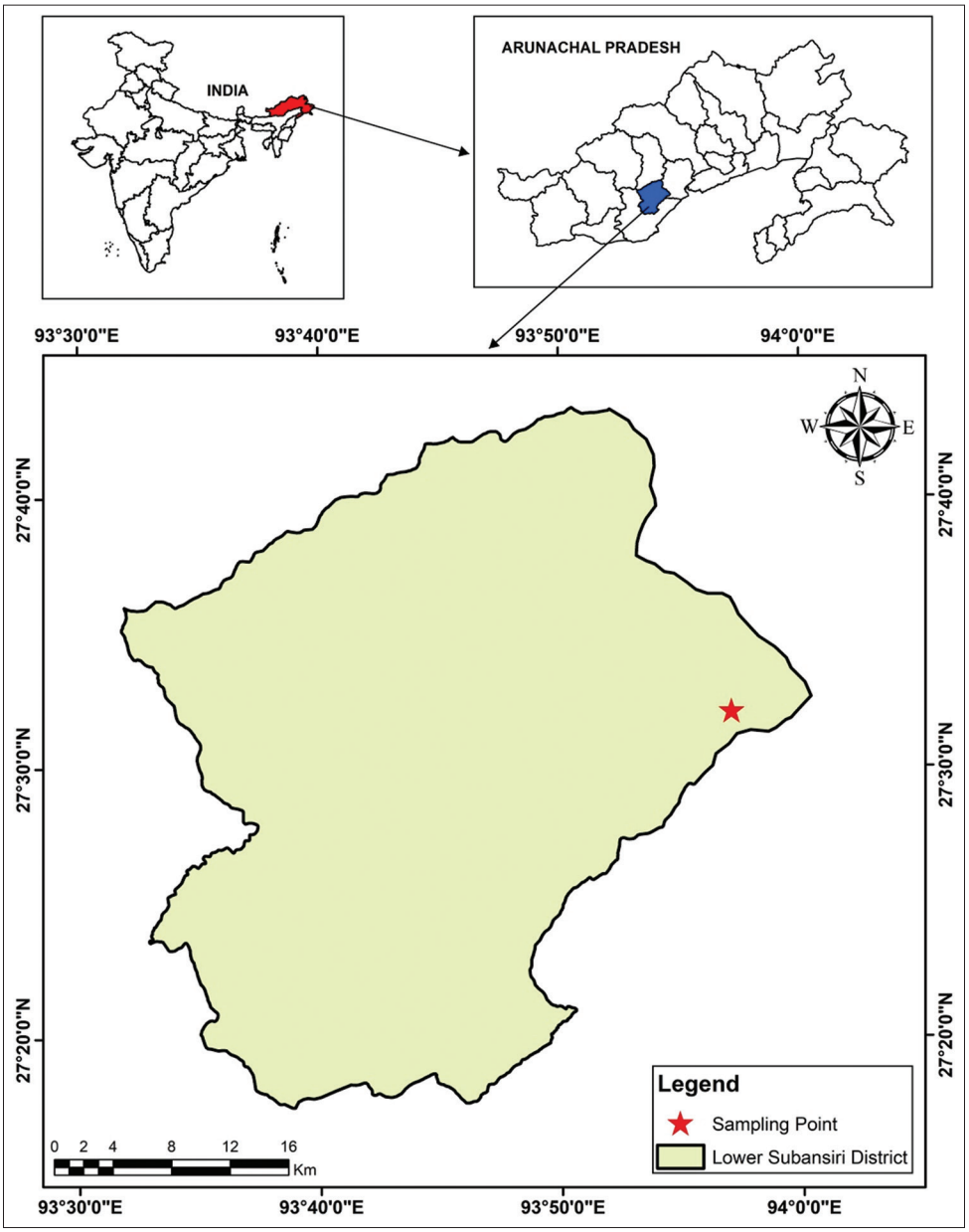

Figure 1: Map of soil sampling site in the Talley Valley Wildlife Sanctuary, Arunachal Pradesh, India.

condition was estimated by measuring the absorbance of the culture at 570 nm using a ultraviolet-visible spectrophotometer (Multiskan Go, Thermo Fisher Scientific).

2.5.2. Effect of the incubation period on the production of violacein

Bacterial cultures were incubated for a total of 7 days. The pigments start producing on the 2nd day of incubation. Violacein production was estimated every 24 h, starting from the 2nd day of incubation [39].

2.5.3. Effect of peptone and beef extract on violacein production

Five different concentrations (3, 4, 5, 6, and 7 g/L) were added to each of the five nutrient broth media to assess the effect of peptone concentrations on pigment production. They were inoculated with 0.02% *C. vaccinii* broth culture at 24 h (v/v). These media (pH 6) were incubated at 30°C. After 7 days, violacein production was estimated. The beef extract in the NBM is a rich source of water-soluble vitamins, carbohydrates, nucleotides, and minerals. Five different concentrations, ranging from 1 to 5 g/L, were added to the nutrient broth to evaluate its effect on pigment production. The inoculation was carried out with 0.02% (v/v) of *C. vaccinii* culture at 24 h. The media were incubated at 30°C for 6 days, and the yield of violacein was measured [40].

2.5.4. Effect of NaCl on the production of violacein

NaCl controls the osmotic pressure of bacterial cells. To evaluate its effect on pigment production, five different concentrations of NaCl were added to the nutrient broth, i.e., 1 to 5 g/L. Each flask was inoculated with 0.02% (v/v) of a 24-h-old *C. vaccinii* culture. The flasks were incubated at 30°C for 6 days, and the violacein yield was estimated [Figure 2a].

The pigment production of bacteria in basal nutrient and optimized nutrient broth media was performed separately by culturing the bacterium in triplicate. The pigment was extracted after 6 days, and the yield of purple-violet pigment was measured.

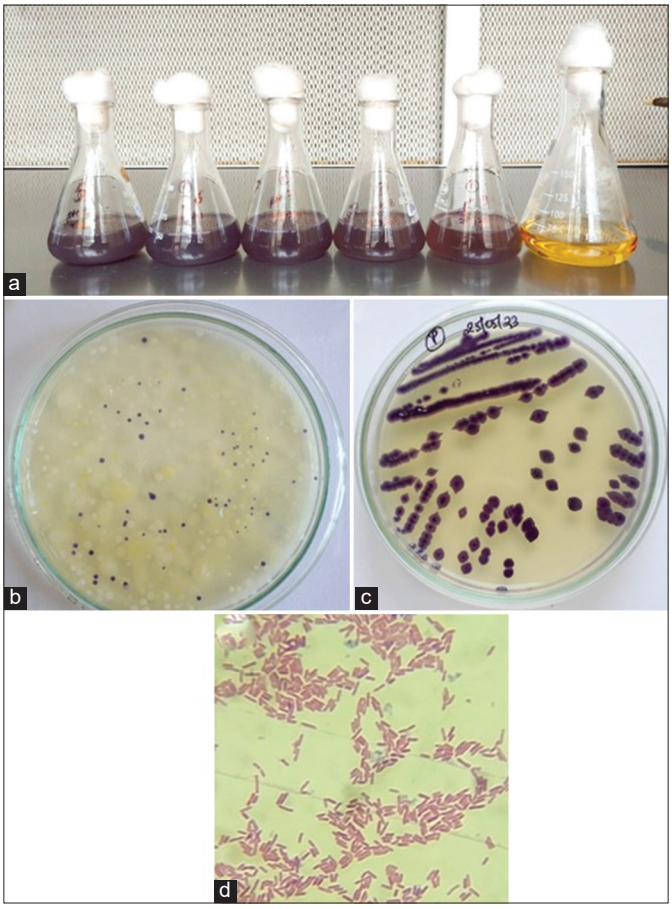

Figure 2: Nutrient broth culture showing production of purple–violet pigment (violacein) in liquid medium as compared to a control medium (a). A mixed nutrient agar plate (b) and pure culture of purple–violet pigment (violacein) producing soil bacterium (c). Microscopic image of Gram -ve rods of *Chromobacterium vaccinii* stained NFML 5214 (d).

2.6. Preparation of Standard Curve of Violacein Pigment

The column fraction of purple–violet pigment (violacein) was used to prepare a standard curve for the determination of pigment concentrations in different pigment optimization experiments. A series of concentrations of 0.1–1.0 mg/mL of purple–violet pigment (violacein) obtained from the column fraction were prepared by dissolving in 1 mL of methanol. The pigment solutions were dissolved properly by vortexing for a few seconds, and 200 µL of each concentration was transferred into a 96-well microplate and absorbance was read at 570 nm using Multiskan Go Spectrophotometer (Thermo Fisher Scientific). A minimum of three replicates were analyzed for each of the different concentrations. The absorbance (optical density) for each concentration was plotted into a scattered graph using MS Excel, and the Y-intercept equation was derived and used for the determination of pigment concentrations in the optimization experiments.

2.7. Evaluation of Textile Dyeing Properties of Violacein Pigment

A simple aqueous dyeing procedure was employed to assess the dyeing potential of the purple–violet pigment using a muslin cloth fabric. A total of 20 g (6.65 g x 3 sets) of bacterial pigment biomass (pellet) was suspended in 50 mL of methanol, and the total volume was adjusted to 100 mL using distilled water and transferred into a heat-resistant glass container. A piece of prewashed muslin cloth (approximately 10 cm × 10 cm) was immersed in the pigmented culture extract solution, and the suspension was heated to boil and kept for 30 min. This allowed the pigment to leak from the biomass into the aqueous medium and simultaneously bind to the fabric. After the dyeing process, the cloth was removed from the solution, rinsed briefly with distilled water to eliminate loosely bound residues, and air-dried at room temperature.

2.8. Statistical Analysis

All the experiments were performed in triplicate, and the results are expressed as the means of triplicate analyses ± standard deviation (SD). One-way analysis of variance was calculated using Origin 7 software to analyze whether the variations in the effect of various nutrient parameters are significant or not.

3. RESULTS

3.1. Morphological Characteristics of *C. vaccinii*

The nutrient agar plates of mixed bacterial colonies and pure culture colonies of purple–violet-colored bacterial isolate are shown in Figure 2b and c. Repeated subculture of the bacterium on NAM and AIA consistently produced purple–violet pigmented colonies of the bacterium. These colonies are circular to sub-elliptical in shape, raised on the surface of the media, and the colony margins are moist and smooth. Microscopic examination revealed that the bacterial cells were Gram-negative rods [Figure 2d]. Based on colony morphological and cellular characteristics, the bacterium is similar to *C. vaccinii;* further, confirmation of bacterial identity was established by 16S rRNA gene sequence analysis.

3.2. Molecular Identification of the Purple Pigment-producing Bacterium

The size of the 16S rRNA-PCR products (L1–L4) are shown in Figure 3. The molecular weight of the PCR amplicons were about 1.4 kb as compared to the molecular ladder (M). The ABI files of both forward and reverse sequences of 16S rRNA gene were assembled, and the consensus sequence (1400 bp, 55% GC) was obtained using Geneious Prime bioinformatics software (https://www.geneious.com/). The sequence was deposited in the GenBank (National Center for Biotechnology Information) with the Accession No. PV613353.1. A DNA sequence homology search was performed using BLASTn (NCBI) in the core nucleotide database (GenBank). The result showed the closest homology and highest identity percentage (99.07%) with the complete genome of *C. vaccinii* strain 21-1 *(*CP017707.1). Based on these results, the bacterial isolate was identified as *C. vaccinii*.

3.3. Molecular Phylogeny of *C. vaccinii* NFML 5214

This molecular phylogeny of *C. vaccinii* NFML 5214 (PV613353.1) was reconstructed in MEGA 12 [41,42] in the presence of 20 numbers of 16S rRNA sequences from 12 *Chromobacterium* species mined from GenBank (NCBI) along with an out-group species, *Serratia marcescens*. The sequences were obtained from the GenBank nucleotide database (NCBI). The reconstructed phylogenetic tree showing the evolutionary relationship of *C. vaccinii* NFML5214 within the genus is shown in Figure 4. Based on the most parsimonious tree, there are two major clades of *Chromobacterium*. Clade I comprises four species, *C. rhizoryzae, C. haemolyticum, C. alkanivorans,* and *C. aquaticum,* whereas Clade II is divided into two sub-clades, IIa and IIb. Clade IIa, consisting of purple pigment-producing *C. vaccinii* of

NFML5214, was separated from clade IIb, consisting of *C. violaceum, C. paludism,* and *C. phragmites*. The phylogenetic tree revealed a close evolutionary relationship between *C. vaccinii* NFML5214 and two other strains reported from sphagnum bogs in the USA (CP017707.1 and NR_109451.1).

3.4. Production of Purple–violet Pigment (Violacein) in Liquid Culture Medium

3.4.1. Effect of temperature

Temperature plays a critical role in influencing bacterial growth and pigment production. The effect of temperature on the pigment production of *C. vaccinii* strain NFML 5214 was studied by incubating the bacterial broth culture at three different temperatures (20, 25, and 30°C). After 7 days of incubation, the pigment yield was estimated by measuring the optical density at 570 nm. The result revealed a positive correlation between temperature and pigment production by increasing from a minimum of 1.25 mg/mL at 20°C to a maximum of 3.69 mg/mL at 30°C, compared to the control medium [Figure 5]. One-way analysis of variance showed significant variation in mean values of the violacein pigment production at different temperatures ($F = 118.665$; $P \leq 0.001$) as shown in Table 1.

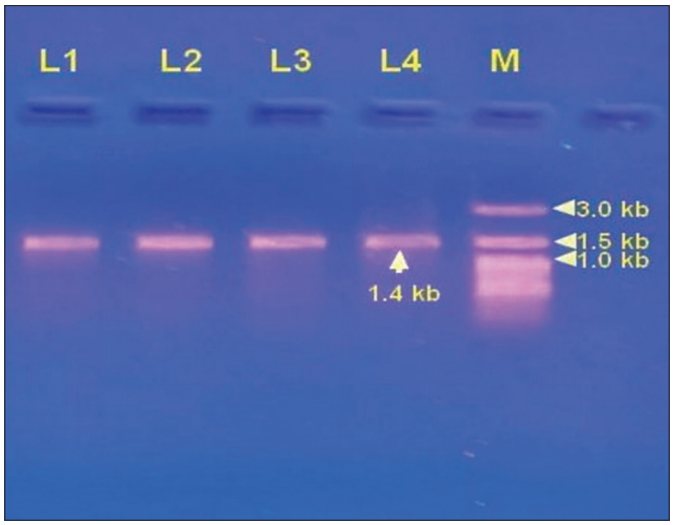

Figure 3: Polymerase chain reaction (PCR) products of 16 rRNA gene of bacterial isolate NFML 5214 on 1.0% agarose gel. PCR amplicons (Lane 1 to 4) and Lane 5 (M) represent 100 bp DNA ladder (MBT130, Hi-Media).

3.4.2. Effect pH on pigment production

To determine the optimal pH of nutrient broth for pigment production, the bacterium was cultured under five different pH conditions (pH 5–9). Initially, the yield of violacein production increased from 2.62 mg/mL at pH 5 to 2.76 mg/mL at pH 6. The yield of violacein then declined from pH 7 to 9, indicating that the *C. vaccinii* strain NFML 5214 produced maximum pigment under moderately acidic conditions [Figure 6]. Significant variation in pigment production was observed at different pH levels of the culture media ($F = 140.763$; $P \leq 0.001$).

3.4.3. Effect of the incubation period on pigment production

The pigment yield was calculated after incubating the cultures for 7 days. The pigment production gradually increased from 0.55 mg/mL on the 2nd day of incubation to a peak of 1.29 mg/mL on the 6th day, followed by a decline from the 7th day onward [Figure 7]. The yield of pigment production was also significantly influenced by incubation period ($F = 23.198$; $P \leq 0.001$).

3.4.4. Effect of media composition on pigment production

Peptone serves as a primary source of organic nitrogen and supplies the required amount of amino acids and proteins for the growth and differentiation of the bacterial culture. As shown in Table 2, an increase in peptone concentration in the nutrient broth was correlated with an

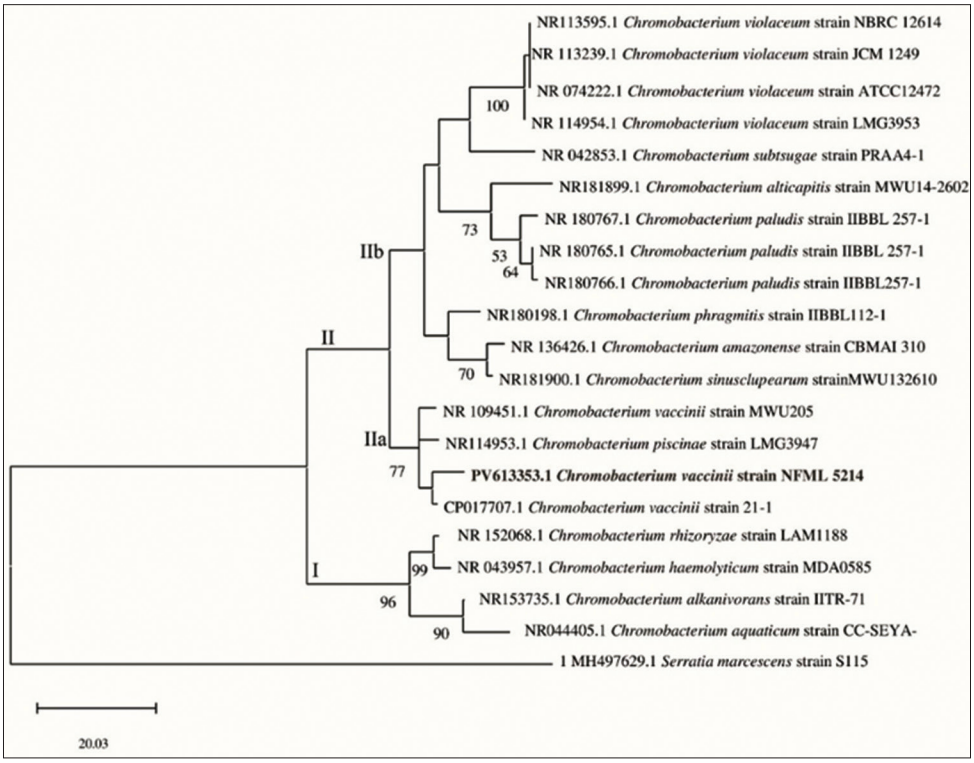

Figure 4: Phylogenetic tree of *Chromobacterium vaccinii* NFML5214.

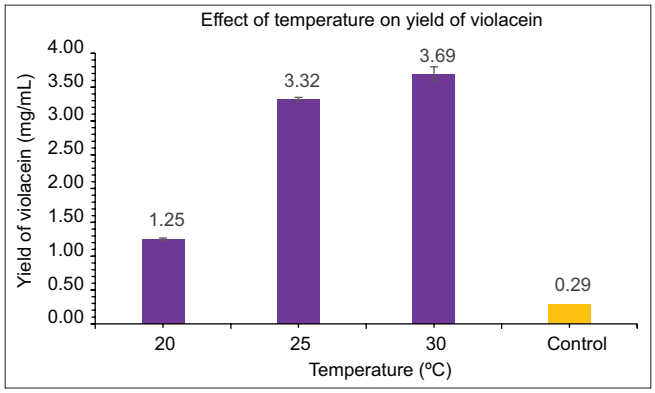

Figure 5: Effect of temperature on pigment production in liquid culture.

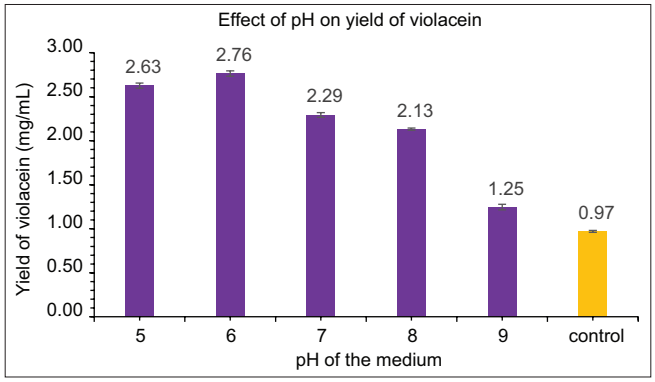

Figure 6: Effect of pH on pigment production in liquid culture.

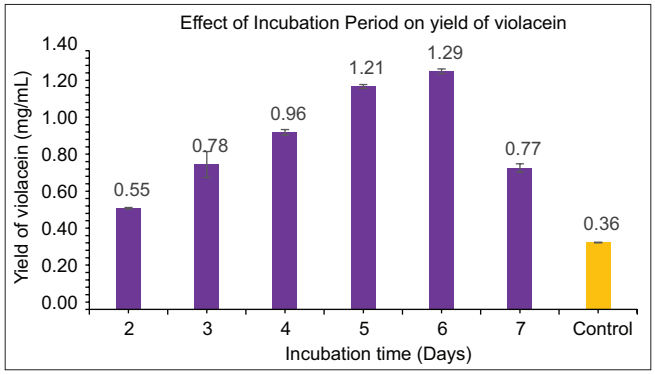

Figure 7: Effect of incubation time on pigment production.

increase in pigment production, with the highest yield observed at 7 g/L with 3.59 mg/mL. Beef extract also provides carbon, nitrogen, vitamins, and trace elements necessary for bacterial growth and helps maintain the osmotic balance of the medium. The highest pigment yield from beef extract supplementation was recorded at a concentration of 4 g/L with 2.11 mg/mL. NaCl maintains the osmotic balance in the medium and influences pigment production. The concentration of 2 g/L NaCl resulted in enhanced production of violacein pigment with 2.50 mg/mL [Table 2]. Different concentrations of three media components, peptone, beef extract, and NaCl also significantly influenced the yield of violacein pigment as revealed by analysis of variance [Table 1].

Table 1: One-way analysis of variance for various parameters used to optimize violacein pigment production.

Parameters	F value	*P* value	Remarks
Peptone (g/L)	267.537	0.00000	Significant
Beef extract (g/L)	12.111	0.00075	Significant
NaCl (g/L)	12.050	0.00077	Significant
pH	140.763	0.00000	Significant
Incubation period (days)	23.198	0.00000	Significant
Temperature	118.665	0.00001	Significant

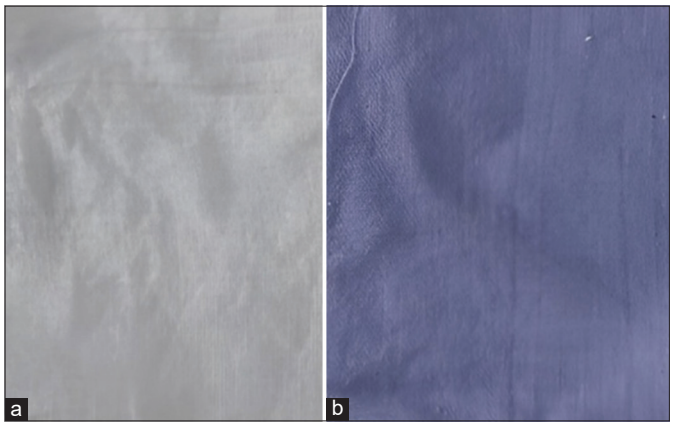

Figure 8: Textile dyeing properties of purple-dyeing pigment (violacein). (a) Undyed muslin cloth and (b) dyed muslin cloth with violacein pigment.

The optimized nutrient concentration medium was evaluated for violacein production. Based on the optimized medium (Peptone [7 g/L], beef extract [4 g/L], NaCl [2 g/L]) incubated at 30°C with pH (6) for 6 days, the maximum yield of violacein pigment was 192 mg/L which was higher than the yield obtained using the basal medium (113.33 mg/L). This result indicates that the optimized medium supported better pigment production, resulting in a higher pigment yield of approximately 69.42%. The increased yield revealed that the optimized medium provided more favorable conditions that enhanced the pigment synthesis efficiency by the bacterium being studied.

3.5. Fabric Dyeing Property of Purple–violet Pigment (Violacein)

Dyeing of the crude aqueous extract of violacein pigment gave the muslin fabric a purple color [Figure 8]. During the dyeing process, the pigment permeated the boiling water and bound to the fabric fibers. After 30 min of boiling, the cloth showed a uniform purple tone with no visible patchiness or uneven dye distribution. The color remained intact after washing, indicating a preliminary level of color fastness under mild conditions. Although no further fastness tests were performed, the color retention after rinsing showed that the pigment has a good natural affinity for the textile.

Table 2: Effect of major nutrient composition on the yield of violacein in liquid culture.

Peptone		Beef Extract		NaCl	
Conc. (g/L)	Yield (mg/mL) ± SD	Conc. (g/L)	Yield (mg/mL) ± SD	Conc. (g/L)	Yield (mg/mL) ± SD
3	2.63±0.006	1	1.80±0.029	1	2.49±0.03
4	2.94±0.019	2	1.89±0.030	**2**	**2.50±0.018**
5	3.05±0.015	3	1.87±0.017	3	2.28±0.04
6	3.39±0.001	**4**	**2.11±0.007**	4	2.16±0.01
7	**3.59±0.012**	5	2.06±0.010	5	2.23±0.01
Temp (°C)	30	Temp (°C)	30	Temp (°C)	30
pH	6.0	pH	6.0	pH	6.0
Time (Days)	6	Time (Days)	6	Time (Days)	6
Control	3.25±003	Control	3.63±0.006	Control	3.37±0.0001

SD: Standard deviation of means

4. DISCUSSION

Chromobacterium species are commonly found in soil and freshwater environments, particularly in tropical and subtropical climatic regions [26,43]. In this study, a purple pigment-producing bacterium, *C. vaccinii* strain NFML5214, was isolated and identified from a rhizosphere soil sample collected from the sub-tropical alpine forest of the TVWS in Arunachal Pradesh, India. The ambient temperature was between 1°C and 2°C at the time of sample collection. This signifies that the species can withstand low atmospheric temperature, as supported by Egorova *et al.*, 2020 [26]. In Bangladesh, five strains of *C. violaceum* have been isolated from water and sediment samples collected and characterized from the Bijoypur white clay Hill Lake in Netrokona [44].

Morphological and molecular approaches were used to identify the bacterium. Bacterial culture on NAM exhibited production of purple–violet colonies. *Chromobacterium* species have continued to attract considerable research interest, primarily due to their ability to produce this characteristic purple–violet pigment [25]. Gram staining revealed rod-shaped Gram-negative bacterial cells. Molecular identification based on 16S rRNA gene sequence analysis exhibited 99.07% similarity to *C. vaccinii* strain 21-1 reported from a sphagnum bog in the USA (CP017707.1). Two species of *Chromobacterium, C. violaceum* (CV4, KJ806351), and *C. vaccinii* (CV5, KJ806485) have been isolated and identified from soil and water ecosystems of Kerala, India, based on morphological and molecular properties (16S rDNA gene sequences) [45]. Another *Chromobacterium* strain (Dyh27s2016) was isolated from the Lake of Manipal International University, and unlike other species of *Chromobacterium*, it was capable of producing Indole [46]. C. *violaceum* capable of producing IAA and enzyme activities (cellulase, xylanase, and protease) was isolated and identified using 16S rRNA gene sequence analysis from the rhizospheric soil of rice fields in Tripura, India [47]. Pigments are secondary metabolites produced by different species of microorganisms, often in response to specific environmental conditions, including stress [48].

Pigment production is affected by both chemical and physical parameters, such as nutrient composition, pH, temperature, and incubation period [10,37,39,40]. In this study, the composition of the nutrient broth was modified to increase pigment production by *C. vaccinii* strain NFML5214. An optimized concentration of peptone (7 g/L), beef extract (4 g/L), and NaCL (2 g/L) in the medium at pH 6, incubated at 30°C for 6 days, produced a maximum yield of purple pigment (192 mg/L) compared to the basal medium (113.33 mg/L). Production of violet purple pigment or violacein by *C. vaccinii* strain NFML5214 in nutrient broth culture as reported in this study is significantly higher than *C. vaccinii* DSM 25150 (3.30–80.86 mg/L) on Luria–Bertani (LB) medium and other agro-waste substrates such as grinded wheat bran, wheat straw, grinded soy cake, and grinded rapeseed cake [49]. The yield of crude violacein pigment produced by *C. vaccinii* strain NFML5214 in the optimized nutrient broth is comparatively higher than that of *C. violaceum* MTCC 2656 strain (125 mg/L) though pigment production in LB was significantly higher (307 mg/L) by *C. violaceum* MTCC 2656 strain [50]. Another study on violacein production by *C. violaceum* MTCC 2656 on submerged nutrient broth and nutrient agar surface culture conditions revealed a higher yield (0.34–0.98 g/L) of crude violacein pigment [51]. In this study, violacein production was found to be maximum under moderately acidic conditions while an increase in pH above 7 led to a decline in pigment production. A similar study revealed the maximum pigment production by a bacterium when the pH of the medium was adjusted to 6.5 [52]. However, other studies have reported that some bacteria produce maximum pigments at neutral pH [10,53]. Interestingly, El Sayed *et al.* [54] reported that the pH of the medium significantly affected pigment production, with the bacteria producing maximum pigment at pH 8. Based on previous reports, several researchers have observed that the optimum pH range for pigment production by the bacteria lies between 6 and 8. Therefore, in the present study, pH optimization was initiated from pH 5 onward, considering that the values below this range are generally unfavorable for pigment biosynthesis. Temperature is also an important factor that influences pigment production in bacteria. In this study, *C. vaccinii* exhibited the highest pigment production at 30°C. This finding agrees with previous reports indicating that the most favorable temperature for maximum pigment production by bacteria is approximately 30°C [10,53]. However, the optimum temperature for maximum pigment production can vary across different bacterial species. For example, *Streptomyces flavofuscus* ARITM02 was reported to produce maximum pigment at a higher temperature of 35°C [55], while *Janthinobacterium lividum* showed peak bluish–purple pigment production at a lower temperature of 25°C [56]. Similarly, *Serratia marcescens* produces the highest pigment yield at 28°C [11].

The structure of violacein comprises two indole moieties, both of which are derived from the amino acid L-tryptophan [57]. The presence of peptone in the nutrient medium provides a slow and sustained release of L-tryptophan to the medium [58], which could have contributed to the enhanced production of purple pigments by *C. vaccinii* NFML5214. Peptone is a better nitrogen source than beef

extract [11]. In this study, pigment production was further enhanced at a lower NaCl concentration of 2 g/L (0.2%). Higher NaCl concentration (0.7%) enhances green pigment production by *B. cereus* [7]. Some strains of *C. violaceum* have been reported to grow and survive at NaCl concentrations as high as 2.5% [46], whereas other *Chromobacterium* species fail to survive in media containing more than 1% NaCl [46]. The incubation period for maximum pigment production yield may vary between species, depending on the metabolism rate of the bacteria and other environmental growth factors. In this study, *C. vaccinii* NFML 5214 exhibited a peak pigment yield on the 6th day of incubation and declined thereafter. A similar study also reported for the *Bacillus* strain, where the yield of red pigment reached its maximum on day 6 and started decreasing after day 7 [59]. In contrast to these findings, *Streptomyces phaeolivaceus* strain GH27 followed a different pattern by producing maximum pigment on the 9th day of incubation and remained constant thereafter [60].

In this study, the successful coloring of muslin fabric with crude bacterial pigment shows the potential of *C. vaccinii* to be used as a natural textile dye even in its crude form. The color remains after rinsing with tap water. This shows that the pigment molecules have a natural attraction to the cellulose fibers in muslin, which is essential for developing sustainable dyeing alternatives. This result aligns with the previous findings of other researchers who reported that the experiment on dyeing both cotton and silk satin fabrics with pigment extracted from *C. violaceum* PDF 23, with colorfastness ratings ranging from fair to excellent [7]. In 2024, Kanade *et al.* used pigments extracted from *C. violaceum* for dyeing different types of fabrics. Their findings show that *C. violaceum* has great potential as a source of natural dye for textile applications. The pigment remained stable and effective at normal body temperature and neutral pH. This natural stability makes it suitable for wearable textiles and emphasizes its promise as an eco-friendly, biologically sourced color for the sustainable textile industry [61]. Ahmed *et al.* highlighted the successful application of violacein as a natural dye for dyeing both natural fibers (cotton and wool) and synthetic materials (nylon and rayon), demonstrating its versatility and potential in sustainable textile dyeing [62].

5. CONCLUSION

Microbial pigments, especially those made by bacteria, have recently received special attention. This is because they are eco-friendly, biodegradable, and non-toxic compared with synthetic dyes. They possess the potential to substitute synthetic dyes in the textile industry. *C. vaccinii* NFML 5214 was isolated and identified using morphological and molecular approaches from the forest soils of TVWS Arunachal Pradesh. The maximum production of the purple–violet pigment (192 mg/L) could be achieved by optimizing the culture medium with 7 g/L peptone, 4 g/L beef extract, and 2 g/L NaCl at pH 6, incubated at 30°C for 6 days. The pigment yield increased by 69.42% in the optimized medium as compared to the basal medium. Since *C. vaccinii* is reported to produce violacein and deoxy-violacein, further studies should be conducted to characterize and identify the pigment and evaluate its biological activities (e.g., antimicrobial, antidiabetic, anticancer, and antioxidant properties) and industrial values (e.g., pharmaceutical dye, textile dyeing, paints), and its suitability as a food dye as an alternative to synthetic dyes.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors' requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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