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ORIGINAL ARTICLE **OPEN ACCESS**

Studies on Violacein Extracted from *Chromobacterium violaceum* with Its Application in Textile Dyeing

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ABSTRACT
Bacteria produce variety of secondary metabolites having pharmacological importance. Chromobacterium violaceum, a Gram-Negative coccobacillus is known to produce a purple color pigment, violacein. The present study focused on the extraction of violacein from Chromobacterium violaceum Bergonzini 1880 MCC 2290[T], its quantitation and application in textile dyeing. The pigment was characterized using Fourier Transform Infrared spectroscopy (FTIR) and High-Resolution Liquid Chromatograph Mass Spectrometer (HRLCMS Orbitrap). The pigment was quantified by using the equation of Beer-Lambert's law. Maximum pigment production of 0.549 mg/ml was obtained after seven days of incubation at 26°C, 120 rpm in nutrient broth adjusted to pH 7. Out of the two methods used for dyeing different fabrics, Simultaneous Fermentation and Dyeing (SFD) gave best results. Tween 80 gave good results when used for washing the dyed fabrics. The dyed fabric showed good antimicrobial activity against Staphylococcus aureus.
Keywords: *Violacein, Chromobacterium violaceum, pigment, textile dyeing.*

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INTRODUCTION

Adverse effects of synthetic pigments on human health and environment increases the demand for natural pigments. Natural pigments can be obtained from plants as well as from microbes. However, instability of the former against light, pH, temperature and non-availability throughout the year makes later a reliable source of natural pigment [4],[13]. Microbes are exploited for the production of their secondary metabolites; pigments are pharmaceutically important ones among them. Being natural, microbial pigments are used as a coloring agent in textile, cosmetics and food industries. Microbial pigments exhibit a vast range of medical applications. Anticancer, antifungal, antibacterial and antiviral properties are few of them [2]. Though many microbial pigments are reported till date, blue and purple bacterial pigments are rare [3]. Violacein is a purple-colored bacterial secondary metabolite produced by the condensation of two tryptophan molecules. It is characterized as $C_{20}H_{13}N_3O_3$. Its molecular mass is 343.3. Violacein is known to produce by various bacterial genera including *Chromobacterium*, *Janthinobacterium*, *Pseudoalteromonas*, *Duganella*, *Collimonas*, *Rugomonas*, *Masillia*, and *Iodobacter*[5]. Production of violacein by *Chromobacterium violaceum* is studied in detail [14]. This bisindole molecule displays different solubility patterns, it is insoluble in water, slightly soluble in ethanol, moderately soluble in dioxane and acetone and soluble in Dimethyl sulphoxoid (DMSO), methanol and ethyl acetate. The UV-Visible spectrum exhibits maximum absorbance at 258, 372 and 575 nm with molar extinction coefficient (Ɛ) of $2.97\pm0.09 \times 10^{-2}$ ml/µg/cm in ethanol [8]. Violacein is slightly soluble in ethanol and soluble in methanol. Alcohols are volatile in nature. Based on these facts we used DMSO as a solution for dissolving the crude pigment extracted from *Chromobacterium violaceum*. When dissolved in DMSO it showed good stability. In this study, we investigated the production of violacein at an interval of 24 h for three days and on seventh day by using the equation of Beer- Lambert's Law. The dyeing property of the pigment was studied on various fabrics. The pH stability, lightfastness and antimicrobial activity of the dyed fabric was studied.

MATERIAL AND METHODS

Microorganism:

Chromobacterium violaceum Bergonzini 1880 MCC 2290[T]procured from National Center for Microbial Resources (NCMR), Pune, Maharashtra, India was used throughout the study. The culture was grown in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth (Himedia) adjusted to pH 7. The culture was grown

by incubating at 26°C and 120 rpm in shaker incubator. The culture was also grown on nutrient agar (Himedia) plates and incubated at 26°C. After 24 h of incubation, the colony characters of the culture were noted. Further, the culture was maintained on nutrient agar slant in refrigerator. Glycerol stocks were prepared and maintained at -22°C. Bacterial inoculums were made by inoculating 24h old colonies of *C. violaceum* from nutrient agar plate in 100 ml nutrient broth and incubated at 26°C and 120 rpm in shaker incubator. Uninoculated sterile nutrient broth was used as a control. For determination of pigment concentration, aliquot of 30ml was removed after every 24 h till 7 days and subjected to the extraction process. After extraction absorbance was measured at $\lambda_{max.}$ All the media used were purchased from Himedia and all the chemical used were of analytical reagent grade.

Extraction of violacein:

Violacein was extracted from *C. violaceum* using some modifications in extraction procedure described by Cortes-Osorio Natalia *et.al*.,2017 as follows: 30 ml aliquots of culture medium taken in the falcon tubes and centrifuged at 12000 rpm for 20 min. Cell free supernatants and bacterial cultures (pellet) were separated. Extraction from the supernatant was performed by mixing it with ethyl acetate and ethanol in 1:1 v/v.Out of the two phases formed, the aqueous (lower) and the organic (top) phase, pigment was obtained by collecting the organic phase. For the extraction of pigment from the pellet, pellets were resuspended in 400 µl Nutrient Broth (NB). Then the cells were lysed using 400 µl sodium lauryl sulphate (SLS) 10% w/v followed by vortexing and incubated at room temperature for 10 min [10]. Extraction was carried out using ethyl acetate and ethanol in 1:1 v/v and centrifugation at 12000 rpm for 10 min. Ethyl acetate was used as organic solvent to obtain violacein crude extracts. The crude extract was then concentrated in hot air oven at 80°C. After drying, the crude extract was dissolved in DMSO and kept at refrigeration temperature[6].

Characterization of extracted pigment:

Crude extract was subjected to the Fourier Transformed Infrared Spectroscopy (FTIR) by scanning wavelength of 500-4500 cm^{-1}. Analysis of results was carried out by manual peak picking method by comparing the peaks with standard functional group charts. Crude pigment extracted after 72 h of incubation was characterized by High Resolution Liquid Chromatography Mass Spectrometry (HRLCMS, Orbitrap) using ESI-MS positive and negative ion acquisition modes (Vanquish). For LC the run time was 35 min. with the flow rate of 300 µl/min. For Orbitrap MS, run time was 0 – 35 min, positive polarity with flow rate of 3 µl/min.

Quantitation of the extracted pigment:

λ_{max} of the crude violacein dissolved in DMSO and ethyl acetate was determined using UV spectrophotometer. For determining the concentration of the extract, 30 ml of broth was aseptically transferred to the sterile centrifuge tube after every 24 h and the pigment was extracted from both supernatant and pellet as described above. Absorbance of the extract dissolved in DMSO was measured at 580 nm. Quantity of the extracts were measured after 24, 48, 72h and 7 days incubation using the equation of Beer-Lambert's law with molar extinction coefficient at 580 nm (ε_{580nm}= 57.856 ml/mg/cm) of crude extract dissolved in DMSO. DMSO was used as a reference.

Dyeing of fabric with crude violacein:

Dyeing property of the pigment was tested on cotton, khadi, synthetic cloth, hosiery and gauze. Two different methods were used for dyeing fabrics mentioned by Kanelli M. *et.al*., [9] with modifications. In first process Simultaneous Fermentation and Dyeing (SFD), sterile 5x5 cm pieces of the selected fabrics were inoculated simultaneously with the *Chromobacterium violaceum* (*C.v.*) culture in a 250 ml Erlenmeyer flask containing sterile nutrient broth (pH 7.4). The flask was inoculated at 26°C at 120 rpm on a rotary shaker incubator for six days. After six days of incubation, the fabric piece was removed and autoclaved. After autoclaving the dyed fabric was washed with 0.1% SLS (w/v) for 1 h at 80°C. Then the fabric was washed with distilled water. In a second method, Dyeing After Fermentation and Sonication (DAFS), 100 ml Sterile nutrient broth in a 250 ml Erlenmeyer flask was inoculated with the *C. v.* culture and incubated at 26°C at 120 rpm on a rotary shaker incubator for six days. After six days of incubation, the broth was sonicated (power 60 for 20 min., 99.9 sec. on and 99.9 sec. off, Ultrasonicater, Bioera) and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was used for dyeing the fabric. Sterile 5x5 cm fabric piece was dipped in the supernatant for 48 h with the addition of 0.02% (w/v) sodium azide in a petri plate of 15mm diameter. After 48 h the fabric was removed and autoclaved. Then the fabric was washed with 0.1% SLS (w/v) for 1 h at 80°C followed by distilled water wash [9].

Stability of the dyed fabric:

In order to mimic the human skin and body conditions, the dyed fabric was dipped in tap water adjusted to pH 3.5 with concentrated Hydrochloric acid and incubated at 37°C for 24 h. Further, effect of different detergents 0.1% SLS (anionic), Tween 80 (neutral) and normal detergent was checked on the dyed fabric. The light fastness of the dyed fabric was checked by directly exposing the fabric to the sun light (summer afternoon sunlight) for 1 h and the intensity of the sunlight was measured with the lux meter.

Antimicrobial activity of the dyed fabric:
Clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Staphylococcus aureus* and *Candida albicans* procured from Armed Force Medical College (AFMC, Pune) were used to check the antimicrobial activity of the dyed fabric. 6 mm disks of the dyed fabric (cotton) were made and autoclaved. 0.1 ml of pathogenic culture was spread plated on sterile Muller Hinton agar (MHA) following 0.5 MacFarland standard. Then the dyed fabric disc was kept at the center of the petri plate with the help of a sterile forcep. The plates were incubated at 37°C for 24 h and checked for zone of inhibition. In another method, after autoclaving, the dyed fabric was simultaneously inoculated in a side arm flask containing *S. aureus* and *E. coli* culture adjusted to 0.5 MacFarland standard. The flasks were incubated at 37°C, 120 rpm on a rotary shaker incubator and O.D. at 620 nm was measured after every 24 h for 3 days.

RESULT AND DISCUSSION
Bacterial growth:
Turbidity observed in the nutrient broth after 24 h of incubation at 26°C, 120 rpm on shaking condition**.** Well isolated violet-colored colonies of the organism obtained after 24 h of incubation at 26°C (Fig.1). Colony characters of the colonies showed Gram negative coccobacilli which were motile.
Extraction of Violacein:
Extracted crude pigment was dissolved initially in three solvents namely ethanol [5], ethyl acetate and DMSO[8,11]. Two different shades of purple color were observed when the pigment was dissolved in ethanol and DMSO (Dark purple) and in ethyl acetate (Brinjal purple) (Fig. 2). 580 nm and 564 nm λ_{max} of the crude violacein dissolved in DMSO and ethyl acetate was found respectively as determined spectrophotometrically. Crude extract in DMSO was used for further studies.
Characterization of Extracted pigment:
Crude violacein was subjected to FTIR studies (Fig. 3). Presence of C-H bond results in the decrease in % of transmittance between 2800-3000 cm^{-1}. The IR bands at 1658.78 cm^{-1}, 1311.59 cm^{-1}, 1018.41 cm^{-1}, 950.91cm^{-1} and 700.16 cm^{-1} correspond to the vibrations of functional groups C=O carbonyl, C-N of indole ring, C-O, C-C and aromatic ring. This evident marks the presence of violacein. All the IR data were in concurrence with literature and standard functional group charts[1,14,15,16]. In the positive ion ESI-MS spectrum, a quasimolar ion peak was observed at m/z 341.3046 (M-H). The MS spectrum of the ion at m/z 341.3046 showed fragmented ions at m/z 101.0032, m/z 157.0352 and m/z 179.0170. All these peaks correspond to the molecular formula of violacein. In the negative ion ESI-MS spectrum, a quasimolar ion peak was observed at m/z 321.2105 (M-H). The MS spectrum of the ion at m/z 321.2105 showed fragmented ions at m/z 160.8420, m/z 265.1480and m/z 293.1794. All these peaks correspond to the molecular formula of deoxyviolacein [15, 16].
Quantitation of violacein:
Concentration of crude violacein extracted after every 24 h till 3 days and after 7 days of incubation at 26°C and 120 rpm as measured by the equation of Beer-Lambert's law using molar absorption coefficient of violacein in DMSO at 580 nm was 0.224 mg/ml, 0.527 mg/ml, 0.413 mg/ml and 0.549 mg/ml respectively. Though after 24 h of incubation, there is not much difference in the pigment production, maximum pigmentation was obtained after 7 days of incubation in nutrient broth adjusted to pH 7 and at 26°C. Result shows similarity with the production of 0.368 mg/ml violacein after 24 h at 25°C, by *J.lividum* in a batch fermentation reported by Kanelli M. *et.al.*, [9]. Choi S. Y.*et. al*., [5] reported slight decrease in the violacein production by *Duganella violaceinigra* YIM 31327 and constant violacein production by *Duganella violaceinigra* str. NI28 after 48 h. R. D. Demoss and N. R. Evans, [7] reported maximum violacein production at pH between 7 and 8 by *C. violaceum* strain ATCC 553 grown on CV (Refers to medium used for production of cells of *Chromobacterium violaceum*) medium.
Dyeing of fabric with crude violacein:
Different fabrics were dyed using SFD and DAFS methods. Out of the two methods used for dyeing, SFD gave best dyeing results than DAFS (Fig. 4). Similar results were reported by Kanelli M. *et.al.*, [9]. Out of the five-fabric tested, cotton and gauze gave best results.
Stability of the dyed fabric:
Dyed fabric showed good stability in acidic condition and at 37°C. Moreover, when the dyed fabric was exposed to direct sunlight of the summer afternoon (1189 Lux intensity measured by the lux meter) no fading of the color was observed. This indicates the stability of the dyed fabric to suit human body temperature, skin pH and climatic conditions. When washed with different detergents, tween 80 showed best results with no color fading followed by 0.1% (w/v) SLS with little fading. With normal detergent washing, cotton, synthetic and hosiery cloths gave good result whereas khadi and gauze showed considerable fading. Further textile parameters like abrasion resistance, color fastness need to be checked.

Antimicrobial activity of the dyed fabric:

All the clinical isolates tested for the antimicrobial activity of the dyed fabric showed little zone of inhibition immediately in the vicinity of the disc. This can be attributed to the size (6 mm) of the dyed fabric disk. Mishra A. *et. al.*,2017 reported inhibitory zone for fabrics dyed with the pigment extracted from *Pseudomonas fluorescens, (Strain, Pf-24)* where the size of the disks used was 28.6 mm[12]. Violacein is water insoluble[8]. This property might have interfered for the pigment diffusion from the dyed fabric[1]. In another method using broth, the dyed fabric showed decrease in the optical density measurement at 620 nm after 48 h for both the test cultures *S.aureus* and *E.coli*. The measurement remains constant for *S.aureus* for further 24 h (72 h). There was little increase in the optical density measurement for *E.coli* after further 24 h (72 h). These results indicate better activity of the dyed fabric against Gram positive organism than against Gram negative ones.

Violacein is a purple gem obtained from different bacteria inhabiting different environmental conditions. This multipurpose pigment needs to be explored more for its use in cosmetics and food industry. Its creditable pharmaceutical properties point out that it can be used for fortification of food apart from a natural coloring agent. Better production strategies and cost-effective media should be developed for the high and cost-effective production of violacein.

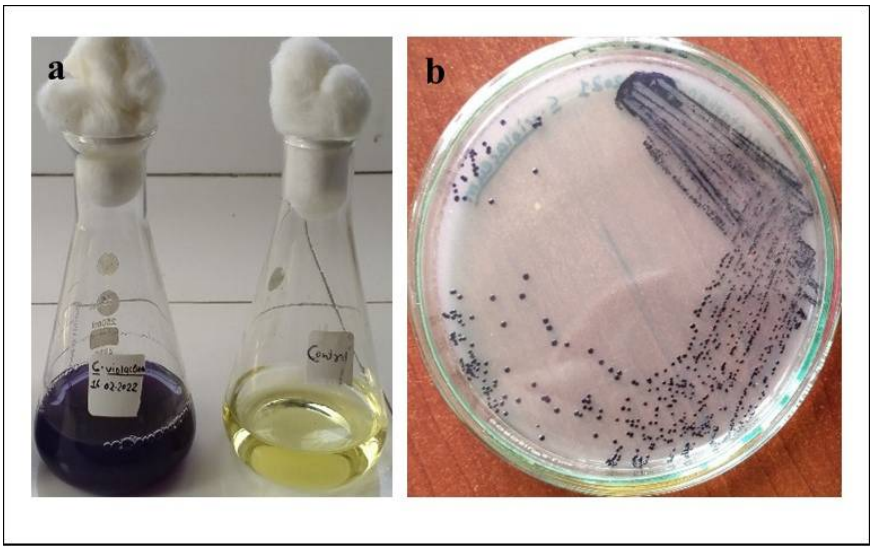

Figure 1. a) Nutrient broth inoculated with *Chromobacterium violaceum* with control b) Violet colored colonies of *Chromobacterium violaceum* on nutrient agar plate.

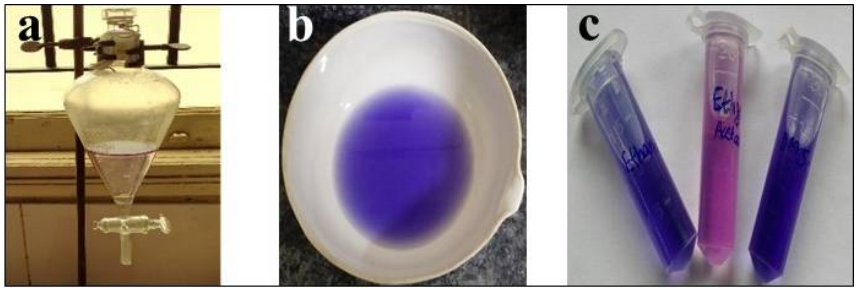

Figure 2. a) Extraction of violacein with separating funnel b) extracted violacein in DMSO c) Violacein in ethanol, ethyl acetate and DMSO.

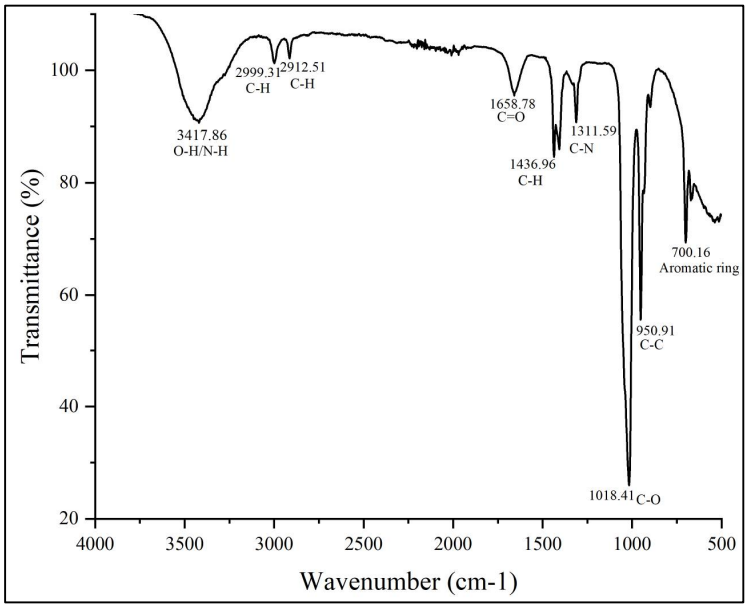

Figure 3: Characterization of crude violacein by FTIR.

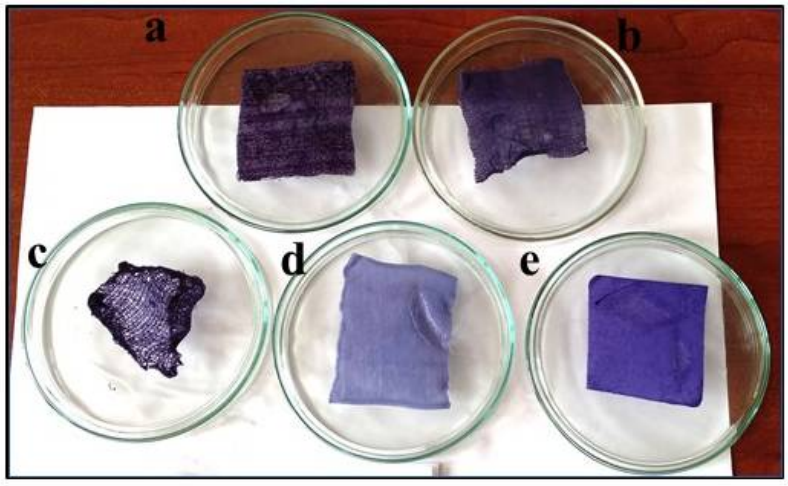

Figure 4: Fabrics dyed by SFD method a) Synthetic b) Khadi c) Gauze d) Hosiery e) Cotton.

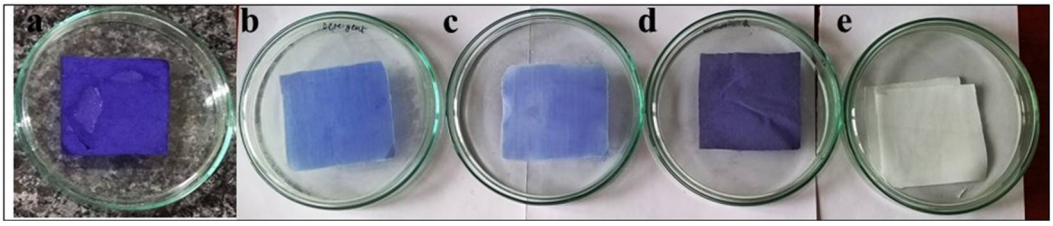

Figure 5: Cotton fabric dyed by SFD method washed with a) Tween 80 b) Normal detergent c) 0.1% SLS d) Unwashed e) Control.

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CONFLICT OF INTEREST

The authors have no conflict of interest to this publication.

REFERENCES

1. Arif S., Batool A., Khalid N., Ahmad I. and Janjua H.A. (2017). Comparative analysis of stability and biological activities of violacein and starch capped silver nanoparticles. *Royal Soci. Chem. Advan.*, 2017;7:4468-4478. DOI: 10.1039/c6ra25806a.
2. Azman A. S., Mawang C.I. and Abubakar S. (2018). Bacterial Pigments: The Bioactivities and as an Alternative for Therapeutic Applications. Nat. Prod. Commun., 13(12):1747-1754.
3. Cardona-Cardona V., Arroyo D., Scellekens J. and Rios-Velazquez C. (2010). Characterization of Blue Pigmented Bacteria Isolated from Puerto Rico. Current Research, Tech. and Edu. Topics in Appl. Microbiol. and Microbial Biotech., Mendez-Vilas Ed.:117-123.
4. Chidambaram K.V. and Perumalsamy L. (2009). An Insightful Overview on Microbial Pigment, Prodigiosin. Elect. J. Biol., 5(3):49-61.
5. Choi S.Y., Kim S., Lyuck S., Kim S.B. and Mitchell R.J. (2015). High-level production of violacein by the newly isolated *Duganella violaceinigra* str. NI28 and its impact on *Staphylococcus aureus*. *Scientific Reports*, 2015;5:15598. DOI: 10.1038/srep15598.
6. Cortes-Osorio N., Cardoso-Cardoso M., Chavarro-Anzola V., Prada-Salcedo L.D., Reyes-Cardenas A., Robles-Camargo J. and Franco-Correa M. (2017). Influence of Environmental Factors on the Production of Violacein Synthesized by *Janthinobacterium lividum*. The Int. J. Engin. and Sci., 6(1):76-83.
7. Demoss R.D. and Evans N.R. (1959). Physiological Aspects of Violacein Biosynthesis in Nonproliferating Cells. J. Bact., 78:583-588.
8. Duran N., Justo G.Z., Ferreira C.V., Melo P.S., Cordi L. and Martins D. (2007). Violacein: Properties and Biological Activities. *Biotech. Appl. Biochem.*, 2007;48:127–133. doi:10.1042/BA20070115.
9. Kanelli M., Mandic M., Kalakona M., Vasilakos S., Kekos D., Nikodinovic-Runic J. and Topakas E. (2018) Microbial Production of Violacein and Process Optimization for Dyeing Polyamide Fabrics with Acquired Antimicrobial Properties. *Front. in Microbiol.*,2018;9:1495. doi: 10.3389/fmicb.2018.01495.
10. Matz C., Deines P., Boenigk J., Arndt H., Eberl L., Kjelleberg S. and Jurgens K. (2004). Impact of Violacein-Producing Bacteria on Survival and Feeding of Bacterivorous Nanoflagellates. *Appl. and Environ. Microbiol.*, 2004;70(3):1593-1599. DOI: 10.1128/AEM.70.3.1593–1599.2004.
11. Menezes C.B.A., Silva B.P., Sousa I.M.O., Ruiz A.L.T.G., Spindola H.M., Cabral E., Eberlin M.N., Tinti S.V., Carvalho J.E., Foglio M.A. and Fantinatti- Garboggini F. (2013). *In Vitro* and *In Vivo* Antitumor Activity of Crude Extracts Obtained from Brazilian *Chromobacterium* sp Isolates. *Brazilian J Med. and Biol. Res.*, 2013;46:65-70. http: //dx.doi.org/10.1590/S0100-879X2012007500167.
12. Mishra A., Jahan S. Singh U.S. (2017). Bio- Coloration of Textile Substrates with Microbial Dye. *Int. Arch. of Appl. Sci. and Tech*., 2017;8[4]:48-53. DOI: .10.15515/iaast.0976-4828.8.4.4853.
13. Navin Raju V. and Radha T. (2015). Production of Extracellular Pigment from Microbes and Its Application. Int. J on Appl. Bioeng., 9(2):23-29.
14. Palukurty M.A., Darsi G.M. and Somalanka S.R. (2019). Effect of Aeration on Growth and Production of Violacein by *Chromobacterium violaceum* using a Bubble Column Reactor. Int. J Manage., Tech. and Engin., 8(12):2903-2910, ISSN NO: 2249-7455.
15. Subramaniam S., Venkatraman R. and Sivasubramanian A. (2014). Synergistic Antimicrobial Profiling of Violacein with Commercial Antibiotics Against Pathogenic Microorganisms. *Pharma. Biol.*, 2014;52(1):86-90. DOI: 10.3109/13880209.2013.815634.
16. Wan A.A., Yusof N.Z., Nordin N., Zakaria Z.A. and Rezali M.F. (2012). Production and Characterization of Violacein by Locally Isolated *Chromobacterium violaceum* Grown in Agricultural Waste. *Appl. Biochem. and Biotech*., 2012;167(5):1220-34. DOI 10.1007/s12010-012-9553-7.

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