

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.907.475>

Antagonistic and Antioxidant efficiency of bacterial pigment isolated from *Chromobacterium violaceum* (BRT-GR2)

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ABSTRACT

Bacterial pigments play an essential role in the defense mechanism of bacterial system against environmental stress. Bacterial pigments are unique secondary metabolite which exhibits various applications such as antibacterial activity against multidrug resistant strains, antioxidant efficiency against radical molecules, anti-inflammatory, anticancerous properties. In the present study, we concentrated on isolating blue pigment producing bacteria *Chromobacterium violaceum* (BRT-GR3). The optimum pH and temperature required for production of pigment with high intensity was found to be pH 8 at 30°C. The presence of violacein was confirmed by the sharp peak at 567 nm. The pigment exhibited wide range of antibiotic spectrum against Gram positive and Gram negative bacteria used in the study. DPPH radical scavenging efficiency of the pigment was found to be increased with increasing concentration.

Keywords

Antibacterial,
Bacterial pigments,
*Chromobacterium
violacein*,
Optimization

Article Info

Accepted:
28 June 2020
Available Online:
10 July 2020

Introduction

Microbes are considered to be the most acquiescing for the production of numerous secondary metabolites such as enzymes, pigments, biosurfactant, amino acids, organic acids, alcohol, and antibiotics. Over other metabolites, microbial colorants have become most essential in the current era due to its wide application in all the fields of science including medicine, photoelectronic,

photosensors and food science (Venil and Lakshmanaperumalsamy 2009). Being colorants, they are widely considered in the manufacture of fabrics and cosmetics with antioxidant and antimicrobial properties. Among the vast group, bacteria play an essential role in the production of pigments by utilizing low cost substrate, shorter incubation time, high yield and feasible extraction procedure (Venil *et al.*, 2014). Bacterial pigments have a become a center of attraction

due to its biological applications such antimicrobial, antioxidant, larvicidal and anticancerous properties. Bacteria possess many properties which make them much easier for handling at large scale production with no seasonal variations (Malik *et al.*, 2012). These bacterial pigments being extracted from biological resource they are less toxic and environment friendly (Cristea and Vilarem 2006). Bacterial pigments obtained from the wild strains are comparatively very less hence to enhance the yield of the wild strain, optimization procedures are highly recommended for better yield at shorter time (Parekh *et al.*, 2000). Carotenoids, Violacein, Prodigiosin, Pyocyanin, Indigoidine, Melanin are the few classic bacterial pigments that were reported to possess various biotechnological applications. Among these, Violacein the blue pigment was reported to possess various application in therapeutics, food and textile industries. Several bacterial species including *Janthinobacterium lividum* (Pantanella *et al.*, 2007), *Chromobacterium violaceum* (Rettori and Duran 1998) and *Pseudoalteromonas luteoviolacea* (Yada *et al.*, 2007) were reported to produce blue pigment. *Chromobacterium violaceum* is a gram-negative soil bacterium which produce violet pigment violacein with diverse applications. Since only very few bacteria produce blue pigment the application, properties and optimization of pigments are inadequate.

In the present study, *Chromobacterium violaceum* is been isolated and identified using microbial and molecular approaches. Optimal conditions required for the growth of bacterial such as temperature, pH and agitation were studied.

Following incubation, pigment extraction was carried out and antibacterial efficiency of the pigment was studied against bacterial pathogens such as *Bacillus subtilis*, *E.coli*,

Klebsiella sp. and *Shigella* sp. Antioxidant efficiency of the pigment was studied using DPPH as free radical.

Materials and Methods

Sample collection and isolation of bacteria

For isolation of chromogenic bacteria, various soil samples were collected from Vellore, district, Tamilnadu, India. The soil samples were collected from the depth of 0.5 cm in a pre-sterilized polythene bag which bears the designation of the sampling site (S1, S2, S3 and S4). The soil samples were transported to laboratory aseptically and stored in refrigerator at 4°C to avoid undesirable microbial contaminations.

Isolation of chromogenic bacteria

The samples collected from various sites were serially diluted using autoclaved water and two fine dilutions were selected for the isolation of the chromogenic colonies. Serially diluted samples were plated over freshly prepared nutrient agar plates and incubated for 48 h at 30° C. Following incubation, plates were observed for the presence of chromogenic colonies and designated as BRT-GR1 to BRT-GR3. The isolates were purified using purification techniques and stored as glycerol stock for further use (Sasidharan *et al.*, 2013).

Identification of chromogenic bacteria BRT-GR3

Based on the intensity and stability of the pigment, BRT-GR3 strain was selected for further study. Microscopic analysis (Colony morphology, and Grams staining) was done to determine the colony morphology and later the isolate was subjected to molecular sequencing for further identification.

16S rDNA sequencing of BRT-GR3

Chromogenic bacterial strain BRT-GR3 was subjected to genomic DNA isolation using genomic DNA isolation kit procured from Himedia. To 5 µl of isolated genome 25 µL of PCR reaction solution (1.5 µL of Forward Primer (8F- AGAGTTTGATCCTGG CTCAG) and Reverse Primer (1541R-AAGGAGGTGATCCAGCCGCA)), 5 µL of deionized water, and 12 µL of Taq Master Mix) were added and PCR were set up with respective conditions. The PCR products were purified and subjected to sequencing. Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis. The sequence was refined manually after crosschecking with the raw data to remove ambiguities and was submitted to the Gene Bank and accession number was obtained.

Optimization for enhancing pigment production

Optimization of bacterial growth factors such as temperature and pH were optimized in order to outline the appropriate condition required by the bacterial to produce maximum pigment.

Effect of temperature on pigment yield

In order to determine the suitable temperature for pigment production, the chromogenic strain BRT-GR3 was subjected to various temperatures such as 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. 50 ml of freshly prepared nutrient broth culture was inoculated with 24 h active culture of BRT-GR3 and incubated at appropriate temperatures for 48 h to determine the influence of temperature of the pigment. The intensity of the pigment was

determined using UV-Visible spectroscopy between 400 to 700.

Effect of pH on pigment yield

Chromogenic bacterial isolate BRT-GR3 was subjected to varying pH for determining the suitable condition for maximizing the pigment production. Freshly prepared nutrient broth culture was inoculated with 24 h active culture and the pH range was altered using NaOH and HCl as pH modifiers. The culture tubes were incubated for 48 h with various pH like acidic (1 and 2), neutral (6,7 and 8) and basic (9,10 and 11). The intensity of the pigment production was determined using UV-Vis spectroscopy between 400 to 700 nm.

Extraction and purification of bacterial pigment

100 ml of freshly prepared nutrient broth culture was inoculated with loopful culture of BRT-GR3 and incubated at optimized culture condition at 150 rpm for 48 h. Prior incubation, the culture media was collected in a sterile centrifuging tube and centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with distilled water to remove the adhered nutrient broth residues. The pellet was further centrifuged and treated with ethanol and incubated for 3 h. Further incubation, the solvent-pellet mixture was centrifuged where, the colored supernatant was collected in a sterile tube and the decolorized pellet was discarded (Asencio *et al.*, 2014).

UV- Visible spectrophotometric analysis of the pigment

To confirm the presence of violacein pigment, one ml of the extracted bacteria pigment was taken and analyzed for the presence of pigment against ethanol as blank. The maximum absorbance range was determined using JASCO V-730 UV-Visible Spectro-

photometry between the range 450 to 700 nm.

Antagonistic ability of bacterial pigment

The extracted bacterial pigment was evaluated for its antagonistic ability against bacterial pathogens such as *Bacillus* sp., *E. coli*, *Klebsiella* sp., and *Shigella* sp. The experiment was carried out on freshly prepared Muller Hinton agar plate using well diffusion method. The wells were made on the agar plate using sterile well cutter. The antagonistic ability of the pigment was demonstrated using varying concentration (20µg/ml to 100 µg/ml). The plates were incubated at 37° C for 24 h. The zone of inhibition around the well was observed and recorded (Kanelli *et al.*, 2018).

Free radical scavenging ability of the pigment

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity of the bio colorant was measured by DPPH method. A solution of DPPH 0.1 mM in ethanol was prepared. 1ml of the solution was added to 3 ml of the pigment fraction in ethanol at different concentrations (100µg to 500µg/ml), mixture was shaken and allowed to stand at room temperature for 1 h (Konzen *et al.*, 2007). The absorbance was taken at 517 nm by using UV Visible spectrophotometer. The percentage of DPPH scavenging effect was calculated using the following equation.

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 is the absorbance of control (Ascorbic acid) and A_1 is the absorbance of test sample (pigment).

Results and Discussion

Isolation of pigment producing bacteria

Among three chromogenic bacterial colonies isolated from the soil samples, a single

bacterial strain was found to produce unique blue color pigment with high intensity. The mixed culture plate has been depicted in Fig 1. The pigment producing strain was designated as BRT- GR3 and preserved as slant at 4° C for further uses (Fig 2). Various study reported the isolation of violet/blue pigment producing bacteria from soil sample. According to earlier study, violet pigment producing psychrotrophic bacteria *Janthinobacterium lividum* XT1 has been isolated from glaciers in China (Lu *et al.*, 2009).

Similarly, *Chromobacterium violaceum* has been isolated from the soils of Kolli hills, Tamil Nadu and studied for its larvicidal efficiency (Baskar and Ignacimuthu 2012). The colony was found to be smooth, shiny, round, convex and pigment producing in nature. The microscopic study shows that the isolate is Gram negative coccobacillus.

16S rDNA sequencing of BRT-GR3

A total of 1432 bases sequence of PCR amplified 16S r DNA gene was determined from the isolate BRT- GR3 (Fig 3) which corresponds to more than 99% of the gene sequence. In the analysis at NCBI and RDP sites it showed homology to 16S r DNA sequences from *Chromobacterium* species.

In the phylogenetic analysis, the sequence formed a cluster within *Chromobacterium* species with 100 % boot strap value, confirming the identity of the isolate as strain of this species. The highest similarity value exists with *Chromobacterium* sp. with the accession number JF734316.1. The bacterial strain BRT- GR3 was identified as *Chromobacterium violaceum* with the NCBI accession ID MF144240. The phylogenetic relationship between the strain BRT- GR3 and other related strains were shown in (Fig 4).

Optimization of culture conditions

Effect of pH and temperature on pigment production

Optimization of culture condition and biomass production was done in order to enhance the production of biomass and secondary metabolite formation. Bacterial strain was subjected to various pH condition in order to determine the maximum yield and the intensity of the bacterial pigment was found to high at pH 8 (Fig 5). Similarly the production of violacein was found to be maximum between pH 7 and 9 (Palukurty *et al.*, 2016). Maximum yield was observed at the temperature of 30 °C (Fig 6). According to the earlier study, the optimum temperature required for maximizing the intensity of the pigment was 25 to 30 °C where, the pigment showed more stability on storage (Venil *et al.*, 2015).

Extraction and characterization of pigment

The 48 h old culture broth was centrifuged and the intercellular pigment was extracted using solvent extraction method (Fig. 7). The absorption spectrum for the ethanolic extract was studied using UV-visible spectrophotometry. The absorption maxima for the

extracted pigment were observed at 567 nm, which was a typical pattern of absorption spectrum of violacein. The sharp peak at indicated the presence of violacein. The obtained UV-Vis spectrum has been shown in Fig8.

Antagonistic ability of pigment from BRT-GR3

The extracted bacterial pigment was evaluated for its antibacterial activity against bacterial pathogens such as *Bacillus subtilis*, *E.coli*, *Klebsiella* sp. and *Shigella* sp.on freshly prepared Muller Hinton agar plate using well diffusion method. The bacterial pigment was tested for its antibacterial activity against bacterial pathogens with varying concentration (20µg/ml to 100µg/ml) (Fig 9). Chloramphenicol was used as positive control and ethanol was used as negative control. At maximum concentration (100µg/ml), *Bacillus* sp. exhibited maximum zone of inhibition of 14mm whereas, *E.coli*, exhibited minimal zone of inhibition(5mm). Similarly, *Janthinobacterium* sp. SMN, an Antarctica bacterium was reported to produce blue pigment and the antagonist activity of the bacteria was found to be wide against gram negative pathogenic bacteria (Asencio *et al.*, 2014).

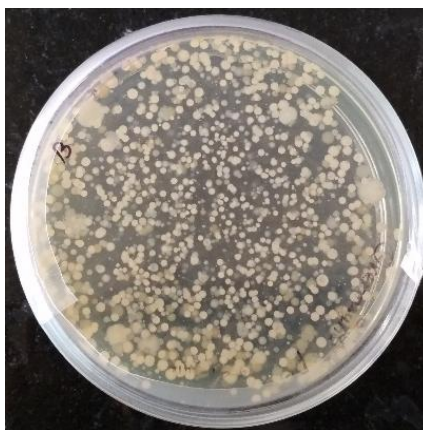


Figure.1 Plate depicting the mixed culture plate of chromogenic bacteria from soil sample



Figure.2 Depicting the pure culture of BRT-GR3

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GCAAGTCGAACGGTAACAGGGTGCTTGACCCGCTGACGAGTGGCGAACGGGTGAGTAATGCC
TCGGAATGTACCGTGTAAATGGGGGATAGCTCGGCGAAAGCCGGATTAATACCGCATAACGCC
TGAGGGGGAAAGCGGGGGATCGAAAGACCTCGCGTTATACGAGCAGCCGACGTCTGATTAGC
TAGTTGGTGAGGTAAGAGCTCACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGATCCG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGAC
AATGGGGGCAACCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGG
ACTTTTGTGAGGGAGGAAATCCCGCTGGTTAATACCCGGCGGGGATGACAGTACCTGAAGAA
TAAGCACCGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGGTGCAGAGCGTTAATCGG
AATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGTCTGATGTGAAAGCCCCGGGCTT
AACCTTGGGAACGGCATTGGGAGACTGCACAGCTAGAGTGCAGAGGGGGGTAGAATTTCC
ACGTGTAGCAGTAAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGG
GATGACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAACTAGCTGTTGGGGGTTTGAATCCTTGGTAGCGTAGCTAACGC
GTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACCTCAAAGGAATTGACGGGGAC
CCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTGCTCTT
GACATGTACGGAACCTTGCCAGAGATGGCTTGGTGCCCGAAAGGGAGCCGTAACACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTTAAGTCCCAGCAACGAGCGCAACCC
TTGTCATTAGTTGCCATCATTAAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAAACCGGAGG
AAGGTGGGGATGACGTCAAGTCTCATGGCCCTTATGAGCAGGGCTTACACGTCATACAAT
GGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCAGAAAAACCGATCGTAGTC
CGGATCGCACTCTGCAACTCGAGTGCCTGAAGTCGGAATCGCTAGTAATCGCAGATCAGCAT
GCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGAGTTTC
ACCAGAAGTGGGTAGGCTAACCGTAAGGAGGCCGCTTACCACGGTGGGATTCATGACTGGGG
TGA
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Figure.3 16S rDNA Sequence data of the isolated strain BRT-GR3

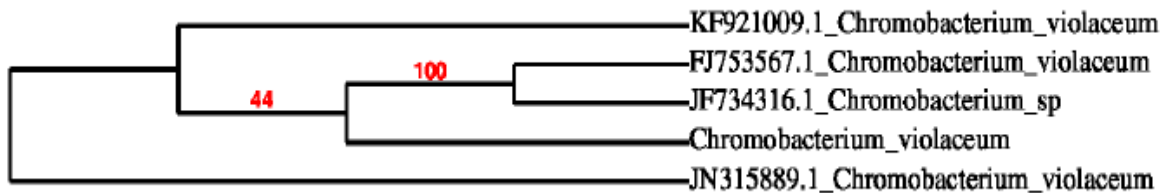


Figure.4 Phylogenetic relationship of the isolate BRT-GR3

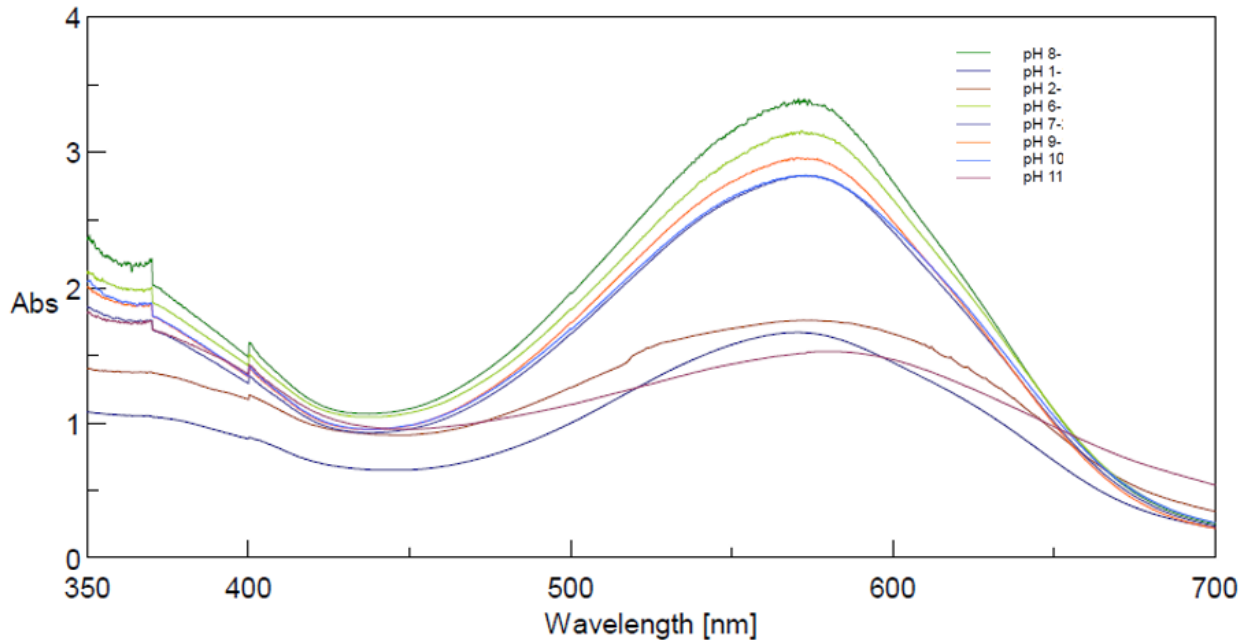


Figure.5 Effect of pH on the intensity of the pigment from BRT-GR2

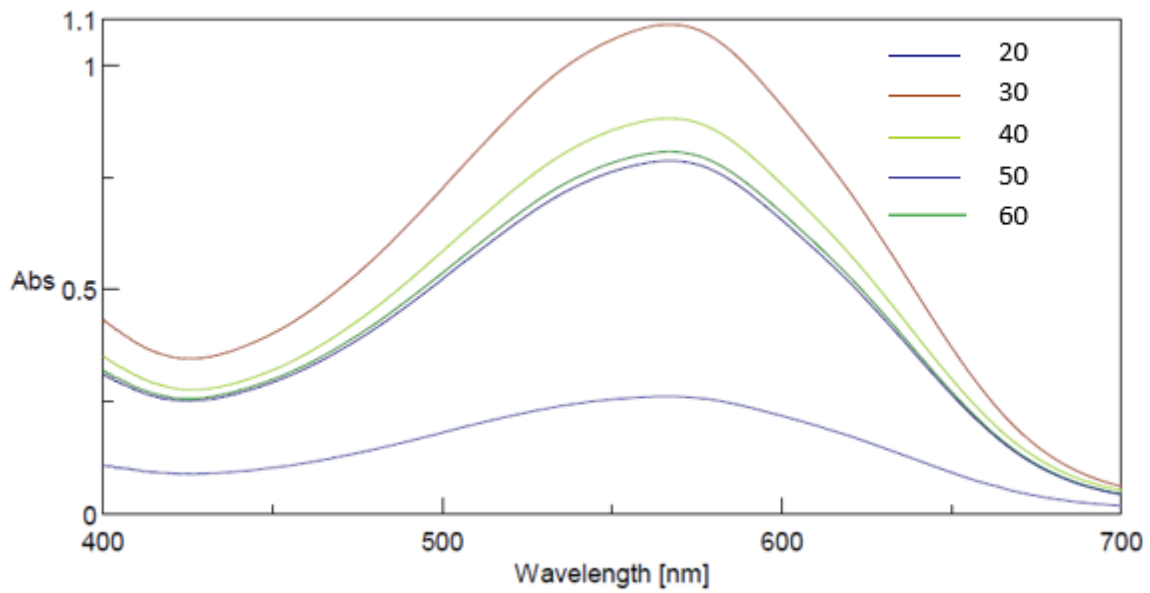


Figure.6 Effect of temperature on the intensity of the pigment from BRT-GR2



Figure.7 Blue pigment extracted from the isolate BRT-GR3

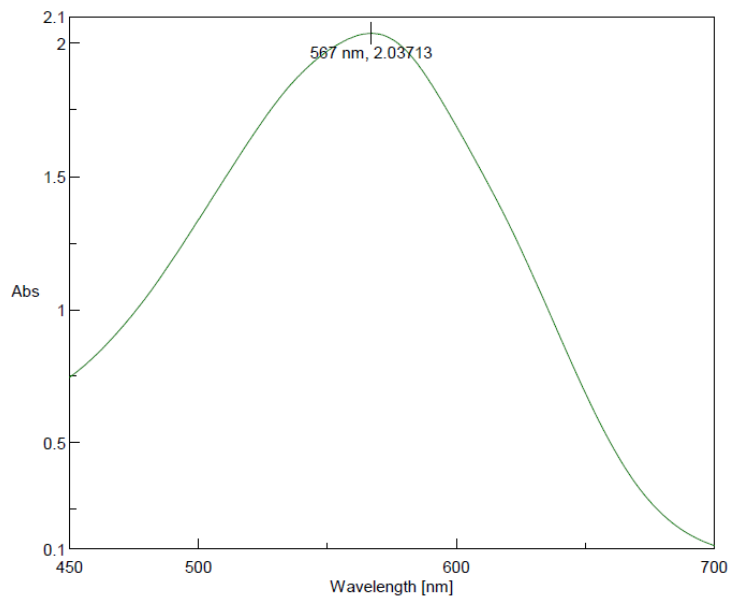


Figure.8 UV-Visible spectra of blue pigment extracted from BRT-GR3

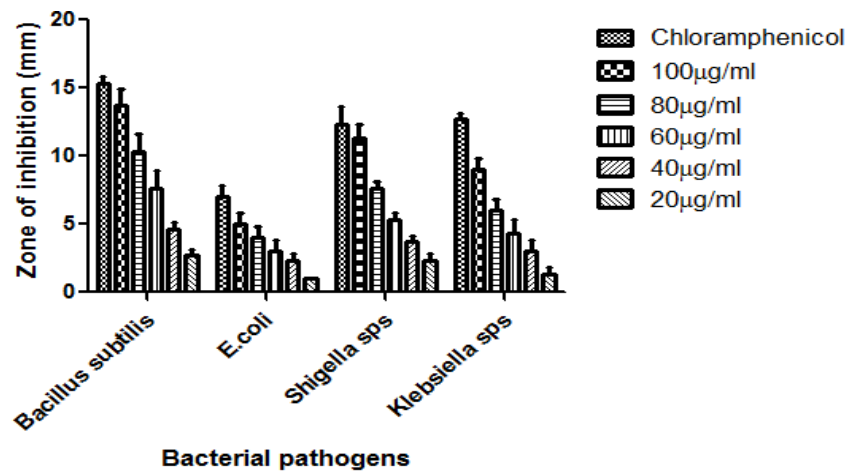


Figure.9 Antagonist activity of bacterial pigment (BRT-GR3) on bacterial pathogens

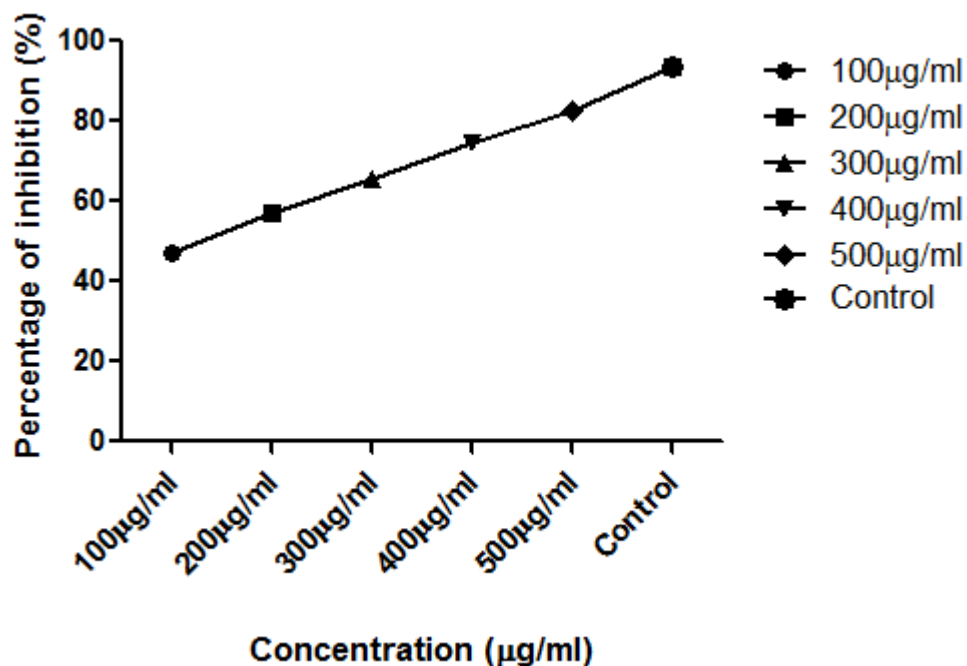


Figure.10 Antioxidant efficiency of pigment extracted from BRT-GR2

Radical scavenging ability of the bacterial pigment

Bacterial pigment was evaluated for its ability to scavenge the free radical scavenging ability. The DPPH radical scavenging activity has been widely used as a model system to investigate the scavenging activity of natural compounds. In the present study, the bacterial pigment showed scavenging activity in a concentration-dependent manner (Fig 10). In DPPH free radical scavenging assay, as the concentration of the pigment increased the percentage of inhibition also increased. According to the previous study, it has been reported that violacein exhibited efficient antioxidant activity (Konzen *et al.*, 2007).

In the present study the chromogenic bacteria isolated from the soil sample was identified as *Chromobacterium violaceum* (BRT-GR3). The optimum pH and temperature required for the production of pigment with high intensity was determined. The UV-Visible spectroscopic data reveals the presence of

Violacein pigment at 567 nm. Antagonistic activity of the pigment was observed to be higher with increasing concentration. Free radical scavenging ability of the pigment was demonstrated against ascorbic acid as positive control where the percentage of inhibition was found to be higher with increasing concentration. Hence, the study concluded that the versatile blue pigment can act as antibacterial agent against multidrug resistant bacteria and scavenge reactive oxygen species even at lower concentration.

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How to cite this article:

Ramya Gunasekaran, Hemapriya Janarthanam and Vijayanand Selvaraj. 2020. Antagonistic and Antioxidant efficiency of bacterial pigment isolated from *Chromobacterium violaceum* (BRT-GR2). *Int.J.Curr.Microbiol.App.Sci*. 9(07): 4050-4059.
doi: <https://doi.org/10.20546/ijcmas.2020.907.475>