Current Research in Microbiology

Chapter 3

Surface Culturing of *Chromobacterium Violaceum* MTCC 2656 for Violacein Production and Prospecting its Bio-Activities

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Abstract

Chromobacterium violaceum MTCC 2656 is a gram negative, facultative anaerobic, non-spore forming bacterium known for its ability to produce violacein, an indole derivative pigment. The pigment violacein was found to possess many potential activities including antimicrobial, anti-cancerous, trypanocidal and antiviral activity. In the present work, effect of carbon sources on pigment production was tested with dextrose, sucrose, lactose and xylose. Pigment production was most favoured in nutrient broth supplemented with 1% dextrose as carbon source when incubated in orbital shaker at 150 rpm for 3 days at room temperature. The violacein pigment was extracted using methanol and ethyl acetate. The obtained violacein pigment was

characterized using UV-Vis spectrophotometer, Infrared spectroscopy and Gas Chromatography –Mass Spectroscopy analysis. The surface culturing method synthesized a significant pigment yield. The pigment was found to inhibit the selected pathogenic bacterial strains. Moreover, the violacein exhibited antioixidant activity. Violacein was found to be a suitable colourant and was able to impart violet colour on recycled paper with high stability.

Keywords: Violacein; *Chromobacterium violaceum;* Pigment; Cheaper substrate

1. Introduction

The increasing awareness and concern on the toxicological effects of chemical colournats had projected the focused of scientific community in prospecting new colourants. Many natural colourants which are eco-friendly and non-toxic have been identified form plants, animals and even from microbial sources. The main issue in utilizing plant based colourants is that it will be extensively harvested for colour extraction leading to vulnerability to extinction. There are many microorganisms with potential for syntheisizing natural colourants. These colourants are very promising with high bio-potential activities and could be produced on large scale. This is hence very advantageous in the commercial application aspects.

Among the potential microbial colourant sources, *Chromobacterium violceium* is worth mentioning. The purple colour pigment violacein synthesized by the bacteria possess many pharmacological applications. This pigment is highly stable and suitable for value addition of many products. Extensive studies were carried out on violacein production using*Chromobacterium violaceum*. Apart from this, there are many reports on other bacterial strains used for the violacein synthesis with different yields and production conditions [1-5].

Violacein is a pigment that is mainly associated with tryptophan metabolism related sendondary metabolite pathway [6]. The complex pathway is critically govern by five enzymes which leads to the violacein synthesis [7]. Violacein production in certain bacterial species like *Pseudoalteromonas tunicata* and *J. lividum* were related to their defense mechanisms against eukaryotic predation and fungal diseases, respectively [8,9].

Pharmacologically, violacein is a potential candidate molecule with numerous bioactivities [1-4,10,11]. These include leishmanicidal, trypanocidal, antifungal, antiviral, antibacterial, antioxidant, antiprotozoal and antitumor activities [1-4]. The pigment was found to significantly inhibit *Staphylococcus aureus* and *E. coli* causing bovine mastitis [11]. Furthermore, synergistic effect for violacein combined with antibiotics like azithromycin and kanamycin against *S.enterica* and s.typhi were also reported [12]. The violacein also possess immunomodulatory potential [13-15], Antioxidant activity [16] and cytotoxicity against breast cancer cell line [17]. Besides the aforementioned activities, violacein also exhibited anti-diarrheal and ulcer-protective effects, immunomodulator, analgesic and antipyretic activity in animal

models [14]. It was also reported to be a photoprotectant [17]. Furthermore, it was also used in textile industry for colouring woven silk fabric with bluish purple color [4].

Chromobacterium violaceum being a commercially known isolate for violacein is well explored. However, the culture conditions are crticial factors which leads to better yield. There is a need for optimization of these isolates to achieve maximum violacein yield. Similarly, the bioactivities of the pigment from different sources may also tend to vary. Hence, this paper mainly focuses on the better violacein production method through surface culturing technique using agar roller bottles. The biopotential activities like antimicrobial and antioxidant potentials of the extracted pigment was validated. The pigment was tested for its application in colouring cellulosic material like recycled papers for making medicated packing material. It could be the pioneering report on stability of violacein pigment and its application potential in paper and pulp industries.

2. Materials and Methods

2.1. Optimization for violcein production

Chromobacterium violaceum culture was procured from NCIM, Chandigarh, India. Cultures were maintained on Nutrient agar plates at 35°C. For the growth and production of violacein pigment, four different carbon sources were tested namely sucrose, dextrose, lactose and xylose. After the production using different carbon sources, absorbance was taken at 586 nm following the solvent extraction method described in the following section.

2.2 Surface culturing of Chromobacterium violaceum

Nutrient agar was supplemented with optimized carbon source for enhancing violacein production. The agar medium was poured into the glass bottle and swirled to get a thin agar film on the inner surface of bottle under aseptic conditions. The roller bottle once set was added with 3-5 ml of 24 hr old culture of *C. violaceum* MTCC 2656. The bottle was shaken vigorously so as to spread the bacterial inoculum on to the agar medium. The agar bottle was incubated upright in an incubator at 35°C for 24 hrs. Violet coloured colonies were observed after the incubation period.

2.3 Extraction of Violacein

Violacein pigment was extracted from the broth using the procedure reported by Sasidharan *et al.* [18] with slight modification. Briefly, 30 ml of broth was added with 10 ml of methanol and incubated for 15 min at shaking condition. 30 ml of methanol lysate was treated with 10 ml of ethyl actetae and mixed well and top organic layer containing violacein pigment was extracted with the help of a separating funnel. The extracts were then concentrated using a rotary evaporator at 50°C, air dried to constant weight and expressed as pigment yield (mg

L^{-1}) [19].

2.4 Violacein Quantification

Violacein production was analyzed by using spectrophotometry as described by Choo *et al*. [20]. 1 mL of 24 hrs old culture was centrifuged at 10,000 rpm for 5 min. The obtained pellet was washed twice with buffer phosphate [pH 7.0]. 1 mL DMSO was added to the pellet and vortexed for 30 s and performed centrifugation as described above. The absorbance of supernatant was read using the spectrophotometer (585 nm) to measure of violacein concentration.

2.5 Purification and Characterization

Intital purification of the crude pigment was passed through silica gel column saturated with methanol. The purified fraction was further concentrated and subjected to TLC using 70% methanol as solvent system [21]. The purified pigment was also characterized using UV-Vis Double Beam spectrophotometer with a scan from range of 400-800 nm. Crystallized violacein was re-dissolved and KBr pelleting was done. This pellet was subjected to FT-IR analysis using Thermonicolet FTIR. GC-MS profiling of the crude extract was performed to identify the various components associated with the major colouring component violacein. For identification of the co-pigments present along with the dominant colouring components, 1 µl of the methanolic fraction was subjected to gas chromatography-mass spectroscopy using THERMO GC- TRACE ULTRA Ver. 5.0 and THERMO MS DSQ II. Helium carrier gas with flow rate of 1 mL min^{-1} was used. The resultant mass spectrum was compared to standards and library search.

2.6 Stability study of Violacein

The stability of pigment during exposure to light and temperature was checked with some modifications in earlier method [22]. For the same, pigment containing vials were incubated at various temperatures and another set exposure to light at different duration. Pigment kept in dark served as control for light stability and pigment at room temperature was used for temperature stability. After the incubation, the vials were visually observed for decrease in intensity and was further confirmed by spectroscopic analysis. Effect of pH change on pigment was also evaluated by adding buffers of different pH. Pigment was subjected to spectroscopic analysis after the shift of pH from neutral to alkaline as well as acid.

2.7 Appication as Natural Colourant

Violceain was identified as a potential colourant for textile industry. Further to exploit this potential, ability of the pigment to colour recycled paper material was evaluated. The recycled paper sheets were designed using the pigment and checked for stability and

photodegradation (fading). Further, L*a*b values of the coloured paper was obtained using Macbeth Densitometer.

2.8 In silico Toxicity analysis

The structure of dominant pigment component violacein was obtained from PubChem. The structure was drawn using Chemsketch software. The *in silico* toxicity of the compound was analysed using LAZAR toxicity predictor and OSIRIS server. Further, metabolite reactive site of the compound was identified using MetaPrint 2D [23,24]. Xenosite prediction of the violacein was also performed to evaluated the residual effect of the compound in environment and its effect over biological system [25-30].

2.9 Antimicrobial Activity of Violcaein

Antimicrobial activity of violacein was carried out using well diffusion method against selected opportunitistic pathogens: *E.coli*, *Klebsiella pneumonia*, *Salmonella typhi* and *Bacillus subtilis*. Various concentrations of violacein pigment (25, 50, 75 and 100 µg) was added into 6 mm wells of Muller-Hinton agar media (HiMedia, Mumbai, India). After 24 hrs incubation at 37±2°C, zone of inhibition was measured and expressed in mm. All experiments were performed in triplicates and values are expressed as mean ± S.D. Microtiter plate assay was also carried out for understanding the percentage of inhibition. OD was read at 600 nm using a microtiter plate reader.

2.10 Antioxidant Activity of Violcaein

The antioxidant activity of the plant extracts was examined on the basis of the scavenging effect on the stable DPPH free radical activity [31]. Ethanolic solution of DPPH (0.05 mM) (300 µl) was added to 40 µl of extract with different concentrations (0.02 - 2 mg/ml). DPPH solution was freshly prepared and kept in the dark at 4°C. Ethanol 96% (2.7 ml) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min and absorbance was measured spectrophotometrically at 517 nm. Ethanol was used to set the absorbance zero. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation [32].

Percentage (%) inhibition of DPPH activity = [(AB– AA) / AB] x 100

Where, AA and AB are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and represented as IC_{50} value for each of the test solutions.

3. Results and Discussion

3.1. Optimization of violacein production

C.violaceum was inoculated into minmal broth supplemented with various percentages of carbon sources like dextrose, sucrose, lactose and xylose. The high yiled was observed in dextrose as carbon source in terms of absorbance **(Figure 1a & 1b)**.

3.2. Surface culturing of *C.violaceum* for violacein production

The surface culture technique was used for cultivation of *C.violaceum*. This technique increase the surface area for microbial mat formation and the inverted sealed bottle provided suitable anaerobic condition which induced the maximum violaein production from *C.violaceum*. The *C.violaceum* colonies were observed as violet mat throughout the agar roll bottle. After the incubation time, the extraction of violacein pigment was performed through methanolic lysis of the cells. The efficient extraction of the pigment was acheieved by repeated methanol lysis. The bottle was vigorously shaken and incubated for 30 min in shaking condition at 120 rpm. Further extraction was carried out by ethyl acetate method. The yield of violacein extracted from surface culturing was about 0.98 g L^{-1} whereas the traditional submereged fermentation yielded only 0.34 g L^{-1}.

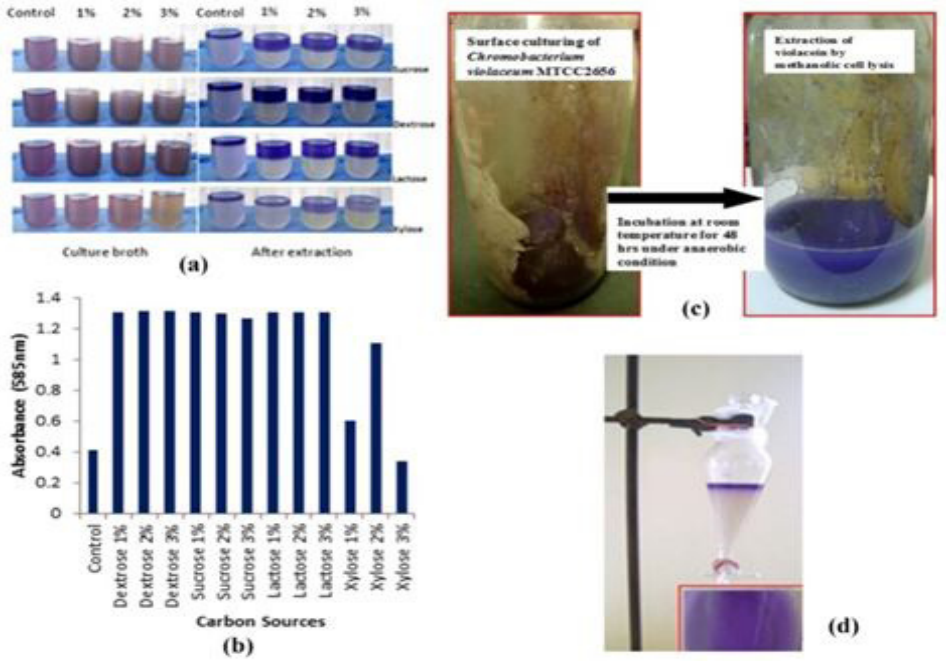

Figure 1: Optimization and production of violacein from *C. violaceum* (a and b) effect of carbon sources on violacein production (c) surface culturing of *C. violaceum* using agar roller bottles and (d) extraction of violacein using separating funnel

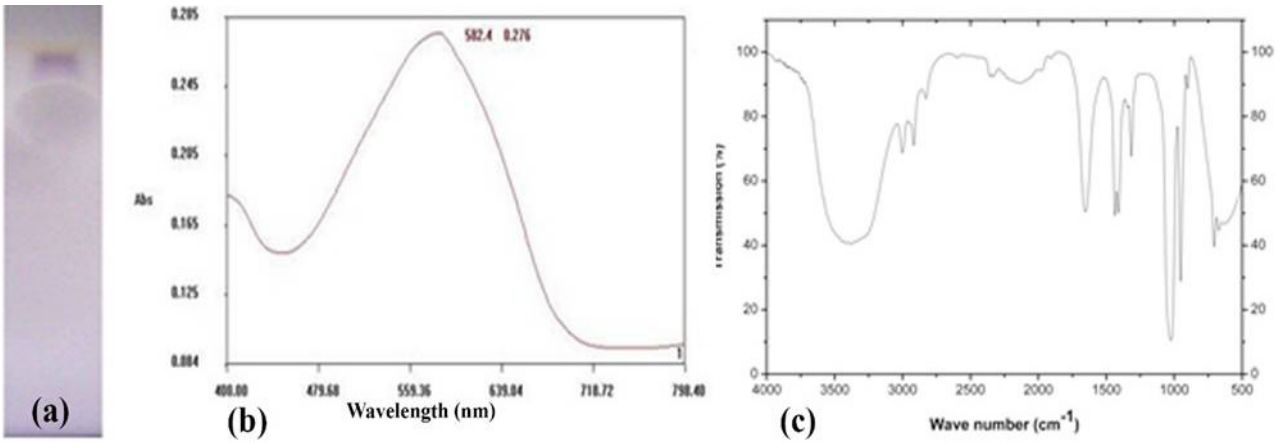

Figure 2: Purification and characterization of violacein from *C. violaceum*. (a) Thin layer chromatogram (b) UV spectrum and (c) FT-IR spectrum of purified fraction

3.3. Purification and characterization of violacein

Following the extraction the violacein content was quntified spectrophotmetrically. Purification of the compound was further carried out using TLC and was compared and confirmed with the Rf values described in previous reports **(Figure 2a)**. The spot was resuspended in methanol and analysed spectroscopically. For violacein methanolic extract Vis λ_{max} (MeOH) (nm): 582 was observed **(Figure 2b)**. In a previous report with different extraction procedure, the violacein-containing butanol phase was varying amounts of a wild-type *C. violaceum* co-scanned over the visible wavelengths of light, violacein produced an absorption spectrum with maximum centered at 585 nm [33]. In the absorption spectrum the pigment showed a maximum absorbance at 571 nm in methanol. It was reported that the UV-VIS spectrum shows strong absorption at the visible region due to resonance of violacein [34].

This purified fraction was subjected to FT-IR spectroscopy. The FT-IR spectrum of the pigment **(Figure 2c)** was analyzed and compared with earlier reports. From the analysis of violacein pigment from IR ν_{max} (KBr) (cm^{-1}) was 706.1 (C-H bending of aromatic compounds), 953.4 (-OH deformation), 1024.0 (C-N vibrations of aromatic tertiary compounds), 1316.1(O-H bending and C-O stretching of primary and secondary alcohols), 1410.4 (C-H bending of alkene), 1436.2(C-H bending of alkanes $–CH_3$), 1654.3(Carbonyl stretching vibrations), 2134.7(N=C=N stretching vibrations), 2917.1, 3001.7 (O-H stretching vibrations) and 3379.8 (N-H stretching vibrations of secondary amines). This result was supported by Aruldass et al.[22].

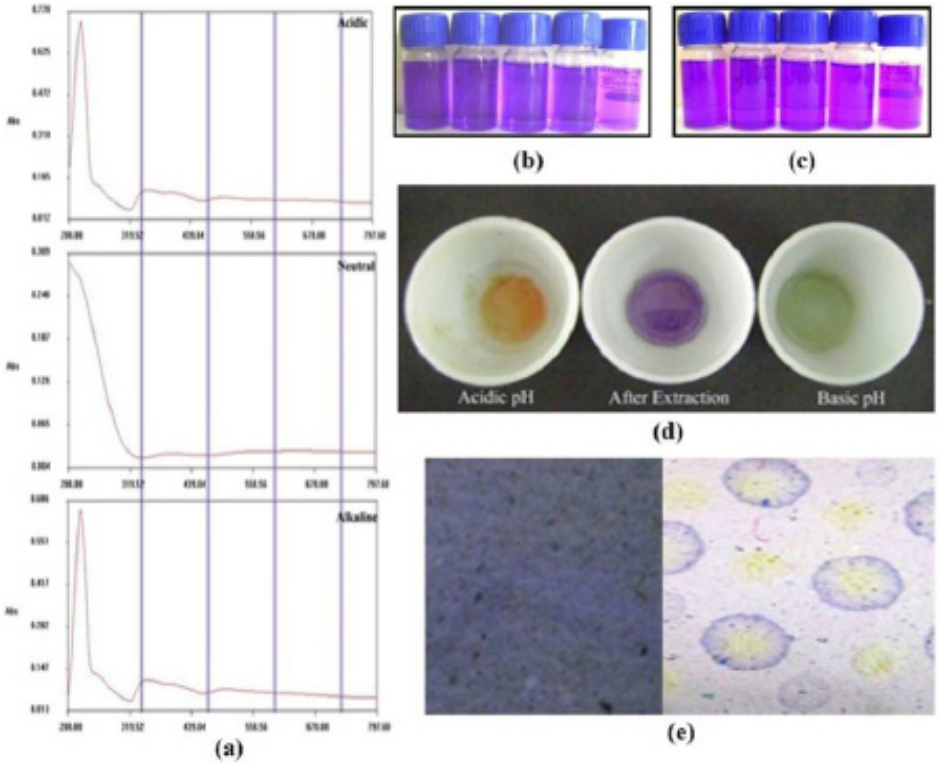

Figure 3: Stability and application of violacein (a) effect of pH on violacein based on UV spectroscopy (b) effect of light and (c) temperature on violacein stability (d) visual observation of violacein colour with pH change and (e) violacein used for colouring and designing recycled paper material

3.4. Stability study of violacein

Effect of pH and light on stability of violacein from *C. violaceum* MTCC 2656 was evaluated. When the crude violacein was made into acidic and alkaline pH, there was an hyrperchromic effect. Similarly, the peaks shifted towards longer wavelengths under acidic and alkaline pH **(Figure 3a)**. The stability of the extracted colourant was studied by exposing to different conditions like temperatures **(Figure 3b)** and photoperiods **(Figure 3c)**. The crude extract of bixin and violacein obtained was stable at temperatures upto 100°C and exposure to light upto a month. L*a*b values for the violacein imparted recycled paper was 41.5 ± 5.64: 0.6 ± 3.77: -10.1 ± 5.80 **(Figure 3e)**. The colour was stable and did not deteriorate after application onto recycled paper surface. The result was in agreement with the previous report of Aruldass *et al*. [22]. The violet pigment exhibited good stability during the entire storage period of 30 days at pH 7, temperature 25–30°C and under dark condition [22].

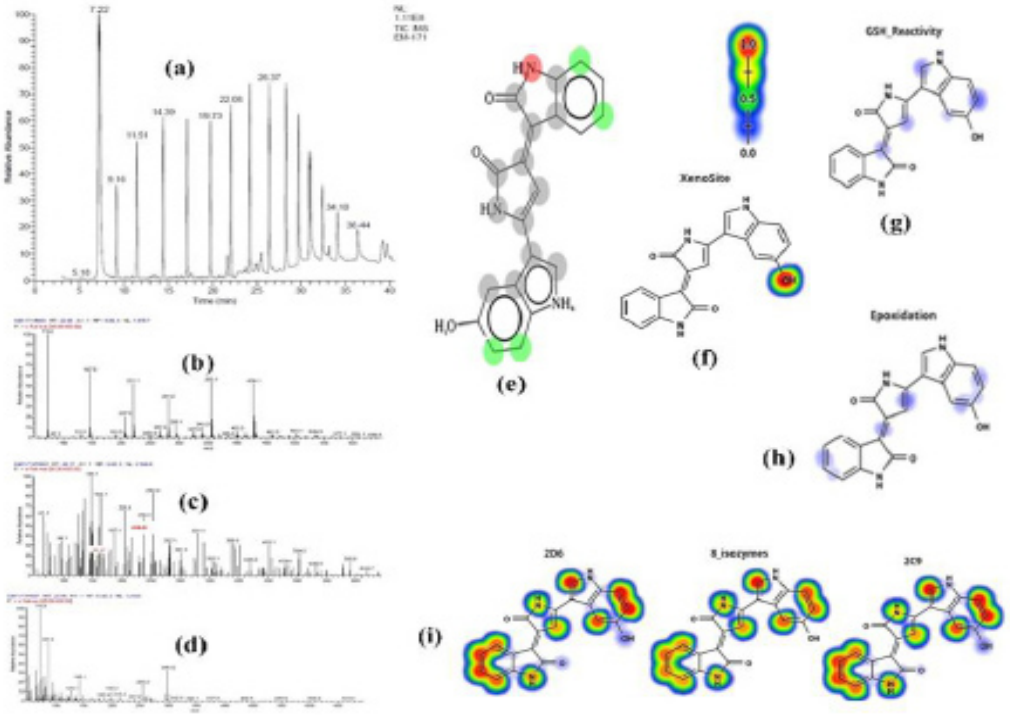

Figure 4: GC-MS Profiling and *in silico* toxicological and phramocological characterization of extract from *C.violaceum* (a) Gas chromatogram showing various metabolites (b-d) Mass spectrum of metabolites (e) Active metabolic reactive site identified in violacein (f-i) Xenosite prediction of violacein

Table 1: Profiling of the extract of *C. violaceum* using GC-MS analysis showing some prominent compounds found other than violacein and its derivatives

Compound Name	Molecular Formula	Molecular Weight	Area %
Di-iso octyl-phthalate	$C_{24}H_{38}O_4$	390	0.40
8'-O-Ethyl-á-Alectoronic Acid	$C_{30}H_{36}O_9$	540	0.40
4-(5-methyl-2-thienyl)but-3-en-2-yl acetate	$C_{11}H_{14}O_2S$	210	0.22
3-Cyano-2,6-diphenyl-4-(trifluoromethylpyridine)	$C_{19}H_{11}F_3N_2$	324	2.14
1-[4'-Aminobutyl]-1-azacyclohexadecane-2,13-dione	$C_{19}H_{36}N_2O_2$	324	2.14

3.5. GC-MS and *In Silico* analysis

GC-MS profiling of the violacein pigment was carried out and various compunds detected were enlisted in **Table 1**. The GC-MS profile of the extract is depicted in **Figure 4 a-d**. The metaprint 2D analysis of violacein revealed the metabolically active sites on the violacein structure **(Figure 4e)**. Xenosite anlaysis was also performed to understand the interaction and residual effects of the compound with biological systems **(Figure 4 f-i)**. This results reveleaed that the copund was suitable for its application in cancerous theraphy. The violacein exhibited low mutagenicity and toxicity based on the LAZAR and OSIRIS toxicity prediction.

3.6. Antimicrobial and Antioxidant activity

Antimicrobial activity of violacein was tested against selected bacterial strains based on microtiter plate method. The extract of various concentrations (50-150 µg) were tested.

There was an increased inhibition percentage with increase in extract concetration. 125 µg of violacein exhibited significant inhibition of all the tested strains compared to the standard antibiotics which was used as the control. The relation with extract concentration and inhibition percentage is depicted in **Figure 5a**. Cazoto *et al*. (2011) reported the antimicrobial efficacy of violacein against *Staphylococcus aureus* isolated from Bovine mastitis. Nakamura *et al*. [35] also reported the antibacterial activity ofthe violet *against Bacillus licheniformis, Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus*, and *Pseudomonas aeruginosa.* Antioxidant activity of the extract was also tested using DPPH method. There was an increased inhibition percentage observed with increase in extract concentration. The extract concentration of 200 µg exhibited more than 50% inhibition **(Figure 5b)**. Konzen *et al*. [16] also established the antioxidant potential of violacein.

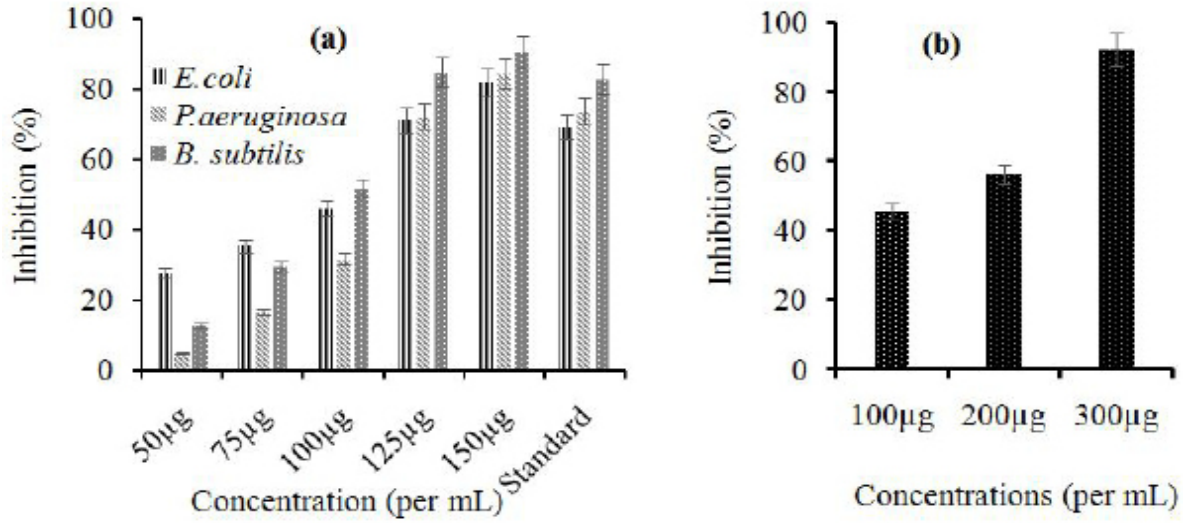

Figure 5: Bioactivities of violacein extracted from *C.violceum* a) antimicrobial activity using microtiter plate against selected pathogens showing inhibition compared to standard antibiotics; and b) antioxidant activity based on DPPH method.

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5. Conclusion

The strain yielded a better violacein content through sucrose supplementation and the surface culture was better in terms of yield. The extracted pigment was satble with biopotentialslke antioxidant and antimicrobial activity against selected pathogens. This work also enlightened on the application of the pigment in paper industries and well as in medicated

packing (antimicrobial) material with good shelf-life and antifading ability.

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