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Violet pigment production from liquid pineapple waste by *Chromobacterium violaceum* UTM5 and evaluation of its bioactivity

Claira Arul Aruldass[a], Rubiyatno[a], Chidambaram Kulandaisamy Venil[a], Wan Azlina Ahmad*[a]

[a]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

Corresponding author
Wan Azlina Ahmad,
Department of Chemistry, Faculty of Science,
Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia
Email: azlina@kimia.fs.utm.my

Highlights

- Liquid pineapple waste, a novel nutritious low cost growth medium.
- Post-treatment of bacterial effluent for eco-friendly disposal.
- Violet pigment stable at optimum conditions.
- Violacein and deoxyviolacein isolated and characterized.
- Crude violet pigment shows bioactivity.
- The first report on the production of violet pigment using liquid pineapple waste medium.

Abstract

Synthetic pigments have been utilized in numerous industries including textile, cosmetic, food and pharmaceuticals. However, the drawbacks of these pigments, namely toxicity problems have kindle the interest in natural pigments. In view of this, the use of natural pigments such as those from bacterial origin offers interesting alternative for industrial application. However, large scale applications of natural pigments are often hindered by the high production cost. This study evaluates on the feasibility of using liquid pineapple waste for the production of violacein by a locally isolated *Chromobacterium violaceum* UTM5 both in shake flask and 50 L bioreactor. The use of optimized growth parameters including culture conditions, concentration of liquid pineapple waste and supplementation of L-tryptophan resulted in violacein yield of 16256 ± 440 mg L^{-1}. Post treatment of the effluent effectively reduced the COD, turbidity and TSS contents to less than 1 mg L^{-1}, 1.57 ± 0.2 NTU and 2.7 ± 0.6 mg L^{-1} respectively. Violet pigment exhibited good stability during the entire storage period of 30 days at pH 7, temperature 25 – 30 °C and under dark condition. The violet pigment has a good antimicrobial activity against selected microorganisms. Of interest, the pigment was active against *Staphylococcus aureus* ATCC 29213 and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 with MIC value of 7.8 and 15.6 µg mL^{-1}, respectively. However, the pigment is toxic to the V79-4 Chinese hamster lung cells with low selectivity index. The purified compounds were determined as violacein and deoxyviolacein using FT-IR, LC-MS and NMR respectively. Results confirmed the feasibility of using liquid pineapple waste as a potential low cost growth medium for large-scale cultivation of violet pigment using *C. violaceum* UTM5.

1 Introduction

Synthetic colours are mostly used in the food processing and cosmetic industries as natural colorants are expensive, less stable and possess lower intensity.[1,2] Conversely, these synthetic colorants have been or being banned due to their carcinogenicity, hyperallergenicity and toxicological issues. Thus, natural pigments are progressively in an increasing demand as they are biodegradable, non-toxic to humans and have precise differences in colour tones.[3,4] A wide range of pigment applications in fields of food, cosmetics, pharmaceuticals and textiles has contributed to its escalating needs by colouring agents in many industries.[1] In comparison to colorants extracted from plant and animals, microorganisms are more attractive sources of pigments due to its production and easy down streaming process.[5] In this sense, representatives of the bacterial genus *Chromobacterium* produce well-known violet pigment that are employed as colorants and has potential medical applications. This pigment is synthesized as secondary metabolites by the biosynthetic mechanism using enzymatic pathway, and at least two structures are recognized: violacein and deoxyviolacein.[6,7] In light of this pigments' potential commercial values, there is a demand to develop high-throughput and cost-effective bioprocesses for pigment production.

In view of high cost of current technology there is a need to develop low cost processes for the production of pigments which could replace the synthetic pigments at industrial scale.[8] Since violet pigment is usually produced in nutrient broth, a novel nutritious and economically cheap medium using agro-industrial residues is desirable to be designed in enhancing the growth of *C. violaceum* and pigment production. Various agro-industrial residues such as rice bran, wheat bran and cassava have been used for pigment production. The utilization of these residues also represents a way of waste management.[9]

In Malaysia, pineapple plantation expands in peat soil area especially in Johor. Malaysia is one of the world major producers other than Thailand, Philippines, Indonesia, Hawaii, Ivory Coast, Kenya, Brazil, Taiwan, Australia, India and South Africa.[10] The production of pineapples in Malaysia increased by 5% from 332,736 MT in 2011 to 335,488 MT in 2012. The revenue generated from the pineapple industry, categorised under tropical fruits production, amounts to RM 110.5.[11] Pineapple is widely consumed as a fresh and canned fruit, as well as in processed juices due to its attractive sweet flavour.[12] Pineapple canning industry is one of many food industries that produced substantial amount of solid and

liquid waste.[13] Pineapple wastes consist of residual pulp, peels, stems and leaves. Regularly, the solid pineapple waste from the cannery industry is sold to the farmers for animal feed or fertilizer.[14] However, the liquid waste is discharged into the nearest stream without an appropriate treatment.[15] Serious environmental problems could occur if these untreated waste disposed to the environment. Since pineapple waste contains valuable components mainly sucrose, glucose, fructose and other nutrients.[13] It is anticipated to use the liquid pineapple waste for industrial process such as fermentation, bioactive component extraction and others. The aim of this study is to develop a fermentation strategy for violet pigment production using liquid pineapple waste and post treatment of effluent in a bioreactor system was carried out. The active compounds present in violet pigment were isolated, purified and characterized. The bioactivity of crude violet pigment was also tested in this study.

2 Materials and methods

2.1 Culture

Chromobacterium violaceum UTM5 (GenBank accession number HM132057), a violet pigmented bacterial strain used in the present study, was isolated from soil sample collected from the vicinity of a wastewater treatment plant in one oil refinery premise in Negeri Sembilan, Malaysia.[16] The culture was grown and maintained by regular subculturing in nutrient broth, NB (Merck, Germany; 8 g L^{-1}).

2.2 Liquid pineapple waste (LPW)

Liquid pineapple waste was collected from Lee Pineapple Co. Ltd. located at Tampoi, Skudai, Johor Bahru. LPW was then filtered using muslin cloth and centrifuged (Allegra^{TM} 25R Centrifuge-Beckman Coulter^{TM}, California) at 10,000 rpm for 10 min at 4 $^{\circ}$C. The supernatant obtained was sterilized using 5 % (v/v) of ethanol and the pH was adjusted to 7.0 using 1 M NaOH prior to use.

2.3 Bacterial growth and pigment production

2.3.1 Effect of culture conditions in Nutrient Broth

Active cultures were prepared by inoculating a loopful of 24 h *C. violaceum* UTM5 into a series of 250 mL Erlenmeyer flasks containing 62.5 mL NB followed by incubation at 30 °C for 24 h in the dark under static and shaking conditions (200 rpm). Then, 10 % (v/v) inoculums were transferred into 125 mL fresh NB medium and incubated under static and shaking conditions (200 rpm). Flasks containing NB (shaking→shaking) acted as control and all the experiments were conducted in triplicates. After 24 h of incubation, violet pigments were extracted using ethyl acetate at a ratio of 4:1 (culture:solvent) and acetone (3 mL) was used to extract the pigments from cells. The pigment was separated from the cells using separating funnel and pigment was then concentrated using rotary evaporator at 50 °C (Büchi, Switzerland). Violet pigment (20 mL) was evaporated to dryness (air dried). The amount of pigment obtained on a dry weight basis was calculated and expressed as pigment yield (mg L^{-1}).

2.3.2 Optimization of parameters for violet pigment production in liquid pineapple waste

The violet pigment production was optimized using one-factor-at-a-time strategy. The potential of using agricultural waste as alternative growth medium was evaluated using liquid pineapple waste as follows; active cultures were prepared by inoculating a loopful of 24 h C. *violaceum* UTM5 into a series of 250 mL Erlenmeyer flasks containing 62.5 mL NB followed by incubation at 30 °C for 24 h in the dark under static condition. Then, 10 % (v/v) inoculum were transferred into 125 mL fresh NB medium and LPW concentrations varying from 2–20 % (v/v; in sterile distilled water), respectively and incubated at 30 °C for 24 h in the dark under shaking condition (200 rpm). The procedure was repeated using NB as control and all the experiments were run in triplicates. The pigment was extracted using ethyl acetate and expressed as pigment yield (mg L^{-1}).

2.3.2.1 Effect of L-tryptophan

Since it was reported that tryptophan simulated violacein production by *Chromobacterium*, the effect in pigment production of L-tryptophan added to the medium was studied.[6,7] Similar experimental procedures as mentioned above were repeated to study the effect of supplementation on the pigment yield. This was carried out using 10–200 mg L^{-1} L-

tryptophan (from 1000 mg L^{-1} stock solution). Non-supplemented medium act as control and all the experiments were run in triplicates. The pigment was extracted using ethyl acetate and expressed as pigment yield (mg L^{-1}).

2.4 Violet pigment production in 50 L bioreactor and post-treatment

Active culture was prepared by inoculating a loopful of 24 h *C. violaceum* UTM5 into a 250 mL Erlenmeyer flask containing 62.5 mL NB followed by incubation at 30 °C for 24 h in the dark under static condition. The starter culture for *C. violaceum* UTM5 was cultivated in static condition by transferring 10 % (v/v) inoculum (active cultures) in a 2 L Erlenmeyer flask containing 500 mL NB and grown at 30 °C for 24 h in the dark. After 24 h incubation, seed cultures were cultivated by transferring 10 % (v/v) inoculum (starter cultures) in 2 L Erlenmeyer flasks (10 flasks) supplemented with 150 mg L^{-1} L- tryptophan (from L-tryptophan stock solution; 1000 mg L^{-1}) at 30 °C for 24 h in the dark under static condition. The 5 L seed culture was then transferred into a 50 L bioreactor (Biotron Liflus GX 75 l, Korea) containing 45 L of 10 % (v/v) LPW, followed by 24 h cultivation under the following conditions: 30 °C, 200 rpm, aeration rate 10 L min^{-1}, initial pH of 7.0, and with addition of Antifoam A (Sigma, Germany). The cell turbidity and violet pigment production were measured every 2 h at OD_{620} and OD_{575}, respectively using a spectrophotometer (Thermo Genesys 20 Spectrophotometer, USA). At 2 h time interval, the viable cell count (CFU mL^{-1}) was determined using spread plate technique. Specific growth rate (μ) is defined as the increase in cell mass per unit time, calculated as follows: $\mu(h^{-1}) = \ln (x_2 \div x_1) \div (t_2 - t_1)$, where x is OD_{620} at t and t is the sampling time.[17] Similarly, specific pigment production rate (μ_p) is defined as the increase in the pigment production per unit time, calculated as follows: $\mu(h^{-1}) = \ln (x_2 - x_1) \div (t_2 - t_1)$, where x is OD_{575} at t and t is the sampling time. Fermentation of *C. violaceum* UTM5 in 50 L bioreactor was carried out three times to ensure reproducibility of the data obtained for yield of violet pigment. The extraction and quantification of violet pigment was conducted as described in Section 2.3.1. The effluent obtained from the pigment extraction was subjected to post-treatment and discharged via the drainage system.

A schematic diagram of the post treatment process of the effluent is depicted in Fig. 2. As seen in Fig. 2, the system has three treatment stages where at the first stage, aeration was introduced to the effluent in 50 L receiving tank using compressor pump for three days continuously. Effluents were pumped by transfer pump to the coagulation tank where colour,

odour and organic contents were removed from the effluent.[18] Polyaluminium chloride (PACl), a coagulation agent with different species distribution was prepared by the batch base-titration method. $PACl_{22}$ and $PACl_{25}$ (OH / Al molar ratio of 2.5 = B value) was prepared at room temperature using the following procedure: 640 mL of 0.5 M $AlCl_3$ was titrated using 1600 mL of 1 M NaOH at 400 mL h^{-1} under rapid stirring. The stirring was ceased with the disappearance of bubbles or when a clear solution was obtained. A volume of 2.5 L of PAC-Al_{13} was filled in the 50 L coagulation tank and effluent was stirred at 120 rpm for 1 h and let to settle for about 2 h before transferred into the holding tank. Sludge formed was compressed and dried as pellets by transferring to a 50 L filter press setup. Slow sand and granular activated carbon was filled in multi-media filter and carbon tank, respectively for filtration process at third stage of post-treatment. The treated effluent was passed through multi-media filter filled with slow sand units and granular activated carbon filter prior to discharge to the nearby water system. The filtered effluent was passed through 0.2/0.4 micron membrane filter to eliminate remaining contaminants before discharging into water. Samples were collected at every treatment stage and centrifuged at 10000 rpm for 15 min. The supernatant was checked for turbidity and chemical oxygen demand (COD) determination using UV-Vis Spectrometer DR5000 (HACH, USA). Meanwhile, the pH was monitored using pH meter (Eutech, Singapore) and total suspended solid (TSS) was monitored using refractometer (Milwaukee, Hungary).

2.5 Evaluation of violet pigment stability

The effect of pH on colour stability of the violet pigment (from LPW) was tested by adjusting the pH of the sample solution using hydrochloric acid (HCl) (1 M and 0.1 M) and sodium hydroxide (NaOH) (1 M and 0.1 M) from 1 to 14. For light stability tests, pigment solutions were incubated under light and dark conditions at room temperature for a month. For temperature stability test, pigment solutions were incubated at 25 °C, 30 °C, 60 °C and 100 °C for a month. All sample solutions were sealed with parafilm to avoid solvent evaporation. The colour change of each solution and hue angle and were measured using UV-Vis spectrophotometer (Thermo Genesys 20 Spectrophotometer, USA) and colour meter with CIELAB colour system (Colorflex EZ colorimeter, United States), respectively.

2.6 Antimircobial activity

2.6.1 Microorganisms

This bioactivity was carried out to evaluate the potency of crude violet pigment as an antimicrobial agent. The test microorganisms were obtained from Centre for Drug Research (CDR), Universiti Sains Malaysia, Penang. The test microorganisms used in this study were as follows: *Staphylococcus aureus* (ATCC 29213), Methicillin-resistant *Staphylococcus aureus* (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603) and *Candida albicans* (ATCC 10231). The strains were maintained on Muller Hinton agar (MHA; Merck, Germany; 34 g L^{-1}) (for bacteria) and potato dextrose agar (PDA; Difco, USA; 39 g L^{-1}) (for fungus) in an anaerobic chamber at 37 °C under atmosphere consisting 10 % CO_2, 10 % H_2O and 80 % N_2.

2.6.2 Minimum inhibition concentration (MIC)

MIC was determined using microdilution method describe by Aruldass *et al.*[19] Commercial antibiotics (vancomycin (Biobasic, Canada) and gentamycin (Biobasic, Canada) for bacteria or amphotericin B (Himedia, India) for fungus) were used as positive controls in this study. Crude violet pigment and antibiotics were diluted in 100 % DMSO (Ajax; Australia) prior to experiment. In 96-well, flat-bottomed microtitre plates, 100 µL of Muller Hinton broth (MHB; Merck, Germany; 21 g L^{-1}) (for bacteria) or potato dextrose broth (PDB; Difco, USA; 24 g L^{-1}) (for fungus) was added. A volume of 100 µL of crude violet pigment was added and serial diluted, to obtain final concentration ranging from 4000 to 3.9 µg mL^{-1}. Final concentration of positive controls ranged from 200 to 0.19 µg mL^{-1}. Finally, wells were inoculated with 100 µL of each microorganism suspension (10^8 cfu mL^{-1}). Each microbial cell suspension was standardized with 0.5 McFarland turbidity standard. The plates were incubated at 37 °C for 24 h for bacteria and 48 h for fungus. Microbial growth was indicated by adding 50 µL of 0.2 mg mL^{-1} of freshly prepared solution of *para* iodonitrotetrazolium (INT; Sigma, Germany) dye in respective plates and reincubated for 30 minutes. MIC was defined as the lowest concentration of extract inhibiting growth of microorganism by preventing colour changes of INT dye in wells from colourless to pink.

2.6.3 Minimum bactericidal/bacteriostatic concentration (MBC)

Subsequently, MBC of crude violet pigment was determined against active microorganisms. MBC was defined as the lowest concentration of pigment that showed complete inhibition of colonies of microorganisms on agar plates. An aliquot of 5 µL of concentrations higher than MIC was cultured on MHA for 24 h at 37 °C. Ratio of MBC/MIC was calculated in order to determine whether the antimicrobial effects were microbicidal or microbiostatic.[19] If the ratio is less than 1, the extract is classified as microbicide and if the ratio is more than 1, the extract is classified as microbiostatic.

2.7 Cytotoxicity

2.7.1 Cell line

V79-4 Chinese hamster lung cells (*Cricetulus griseus,* V79-4, CCL-93^T) were purchased from American Type Culture Collection (ATCC). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) maintained at 37 °C in 5 % CO_2 and 95 % air by standard culture techniques. Cultures were examined daily to ensure they remain healthy.

2.7.2 MTT reduction assay

The cytotoxicity of the crude violet pigment against V79-4 Chinese hamster lung cells (*Cricetulus griseus,* V79-4, CCL-93^T) was assessed by the MTT reduction assay[20,21] with slight modifications. The V79-4 cells were seeded at a density of 10,000 cells (100 µL) in each well of 96-well microtitre plates and incubated at 37 °C and 5 % CO_2 incubator. A stock solution of violet pigment (200 mg mL^{-1}) was prepared in 100 % dimethyl sulfoxide (DMSO; Ajax, Australia). After 24 h incubation, crude violet pigment (100 µL) at varying concentrations ranging from 62.5, 125, 250, 500, 1000 and 2000 µg mL^{-1} in complete DMEM medium (supplemented with 10 % FBS) were added to the wells and the control cells were treated with 1 % (v/v) DMSO. The plates were incubated for 24 h in a 37 °C and 5 % CO_2 incubator. After incubation, 30 uL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg mL^{-1}) in phosphate buffer saline (PBS) (MTT; Sigma, Germany) was added to each well and the plates were incubated in a 37 °C and 5 % CO_2 incubator for 4 h. DMEM mediums were aspirated from the wells and 200 µL of PBS was added to rinse each well. A volume of 200 uL of DMSO was added into each well to solubilize the purple formazan crystals. The absorbance was measured using microplate reader at 570 nm. Cell growth inhibition for violet pigment was expressed in terms of LC_{50} values, defined as the

concentration that caused 50 % of inhibition of cell viability. Each experiment was repeated in triplicate. Selectivity index values were calculated by dividing cytotoxicity LC_{50} values by the MIC values of violet pigment (SI = LC_{50}/MIC).

2.8 Purification and characterization of violacein and deoxyviolacein

For purification of violacein and deoxyviolacein, the violet pigment obtained from ethyl acetate extraction was subjected to a silica gel vacuum liquid chromatography (VLC; 60 g (Silica gel 60 (0.04-0.063 mm), Merck, Germany), column size: 7.0 cm × 5.0 cm, solvent system: chloroform, chloroform-acetone, in the order of increasing polarity) to give 40 fractions (50 mL each). Each fraction was subjected to TLC analysis. Fractions with similar pattern on TLC were combined to give three major fractions (yellow, orange and violet). The violet fraction was further purified by preparative thin layer chromatography (Silica gel 60 PF 254 containing gypsum, Merck, Germany; plates: 20 × 20 cm) with a solvent system of chloroform: acetone (5:5). Two purple bands were observed with the R_f value of 0.43 and 0.58 and suggested as violacein **(1)** (Fig. 1) and deoxyviolacein **(2)** (Fig. 1), respectively.[16] The FT-IR spectrum of the **(1)** and **(2)** were recorded with a spectrometer (Perkin Elmer, USA). FT-IR absorption for **(1)** in KBr was at ν_{max} 3421 (s), 3237 (br), 1689 (s), 1669 (s), 1621 (s), 1279 (s), 1219 cm^{-1} (s). FT-IR absorption for **(2)** in KBr was at ν_{max} 3425 (br s), 1670 (br w), 1620 (br w), 1279 (br w), 1214 cm^{-1} (br w). The molecular mass of both compound on ESI-MS were 343 $(M-H)^-$ and 327 $(M+H)^+$, which correspond to that of **(1)** ($C_{20}H_{13}N_3O_3$) and **(2)** ($C_{20}H_{13}N_3O_2$), respectively. The purified **(1)** and **(2)** were dissolved in deuterated DMSO (Merck, Germany), ^1H and ^{13}C (DEPT Q) spectra were recorded using 400 and 100 MHz NMR (Bruker, Germany), respectively.

The NMR chemical shifts of **(1)** were as follows: ^1H- NMR (DMSO, 400 MHz, ppm) δ 6.79, (1H, dd, *J*=8.4, 2.4, H-6′); 6.82 (1H, d, *J*=7.6, H-7″), 6.95 (1H, td, *J*=8.8, 1.2, H-5″), 7.20 (1H, td, *J*=7.6, 0.8, H-6″), 7.24 (1H, d, *J*=2, H-4′), 7.35 (1H, d, *J*=8.8, H-7′), 7.55 (1H, d, *J*=2, H-3), 8.07 (1H, d, *J*=3.2, H-2′), 8.93 (1H, d, *J*=8, H-4″), 9.35 (1H, s, 5′-OH), 10.63 (1H, s, NH), 10.75 (1H, d, *J*=1.6, NH″) and 11.90 (1H, d, *J*=2.8, NH′). ^{13}C- NMR (DMSO, 100 MHz, ppm) δ 97.4 (C-3), 105.0 (C-4′), 106.2 (C-3′), 109.5 (C-7″), 113.6 (C-6′), 113.9 (C-7′), 119.2 (C-4), 121.3 (C-5″), 122.8 (C-3″a), 126.1 (C-3′a), 126.8 (C-4″), 129.9 (C-2′), 130.1 (C-6″), 132.1 (C-7′a), 137.4 (C-7″a), 142.3 (C-3″), 148.0 (C-2), 153.4 (C-5′-OH), 170.7 (C=O) and 172.1 (C=O).

The NMR chemical shifts of **(2)** were as follows: ^{1}H- NMR (DMSO, 400 MHz, ppm) δ 6.84, (1H, d, *J*=7.6, H-7″); 6.97 (1H, td, *J*=8.0, 0.8, H-5″), 7.23 (1H, td, *J*=7.6, 1.2, H-6″), 7.31 (2H, m, H-6′), 7.57 (1H, m, H-7′), 7.66 (1H, d, *J*=2, H-3), 7.85 (1H, m, H-4′), 8.21 (1H, s, H-2′), 8.95 (1H, d, *J*=7.6, H-4″), 10.66 (1H, s, NH), 10.83 (1H, d, *J*=2.8, NH″) and 12.14 (1H, s, NH′). ^{13}C- NMR (DMSO, 100 MHz, ppm) δ 97.9 (C-3), 106.8 (C-3′), 109.6 (C-7″), 113.4 (C-7′), 120.0 (C-4), 120.2 (C-4′), 121.4 (C-5″), 122.1 (C-6′), 122.7 (C-3″a), 123.6 (C-5′), 125.0 (C-3′a), 127.0 (C-4″), 129.9 (C-2′), 130.3 (C-6″), 137.2 (C-7″a), 137.9 (C-7′a), 142.5 (C-3″), 147.5 (C-2), 170.6 (C=O) and 172.0 (C=O).

2.8 Data analysis

All the results were presented as mean ± standard deviation. Data were analyzed by t-test for the determination of statistical significance between groups. *p* values of 0.05 or less were considered significant.

3 Results and discussion

3.1 Effect of culture condition on pigment production

The effect of different condition on the production of violet pigment by *C. violaceum* UTM5 is presented in Table 1. Highest pigment yield of 258 ± 8 mg L^{-1} (p<0.001) was obtained as *C. violaceum* UTM5 in NB was changed from static to shaking condition. Yang *et al.*[22] reported that *Pseudoalteromonas luteoviolacea* produced high amount of violet pigment (violacein) under static compared to shaking condition. The effect of shear force during high agitation rate decreases the pigment production.[23,24] In the present study, initial adaptation of bacteria to the media might occur in static condition. Occurrence of minimal shear stress may increase the violet pigment yield, as the inoculum was further cultivated in shaking condition. Continuous cultivation of *C. violaceum* UTM5 in static condition produced significantly lowest pigment yield of 170 ± 9 mg L^{-1} (p<0.05). It was reported that agitation rate influences pigment (violacein) production from *Pseudoalteromonas luteoviolacea*.[22] At static and low agitation conditions, cells formed clusters and became increasingly separated into single cells when grown in higher agitation. This may interrupt the aggregation of cells because of shear stress provided by the shaking motion. Agitation, aeration and shear stress were found to be the key factors in metabolite production in microorganism.[25] It was found that higher aeration and agitation caused higher shear stress and those could lead to cell death rates, eventually affecting the metabolite production. Thus, in this study continuous shaking may increase the

amount of shear stress in *C. violaceum* UTM5 and eventually suppress the violet pigment production. In the first step of violacein biosynthesis, molecular oxygen was reported to be an essential factor during hydroxylation of tryptophan with the production of an intermediate 5-hydroxytryptophan.[26,27] However, this study showed that less pigment was produced during continuous shaking, implying that the concentration of dissolved oxygen may not have any effect on pigment production in *C. violaceum* UTM5.

3.2 Effect of liquid pineapple waste concentration on pigment production

From this study, the yield of pigment significantly increased as the concentration of liquid pineapple waste increased (Fig 3). A maximum yield of 285 ± 5 mg L^{-1} (p<0.001) was achieved at 10 % (v/v) of liquid pineapple waste and the pigment yield gradually decreases for subsequent concentration of liquid pineapple waste. The maximum yield was slightly higher than the yield obtained in nutrient broth (239 ± 3 mg L^{-1}). LPW that is used in this study was previously characterized by Othman *et al.*[13] and reported to have high sugar contents, namely sucrose, glucose and fructose, monovalent (postassium) and divalent cations such as magnesium, calcium, and other elements including iron, manganese, zinc, copper, cadmium and sodium. Thus, these substances in LPW provide a suitable condition for growth of *C. violaceum* UTM5 and act as natural carbon sources for the pigment production. It was also reported that LPW comprises of anion compositions including chloride phosphate, sulphate and nitrate ions.[13] Since chloride ion is the principle extracellular anion in any organism, *C. violaceum* UTM5 is able to utilize this ion for its growth. Other anions were used as energy sources during its cultivation.[13]

Other report also mentioned the presence of organic substances in pineapple wastes which are employed as carbon and nitrogen sources for cell growth and by-product production.[28] Kurosumi *et al.*[29] suggested pineapple waste as a source of carbon for bacterial production of cellulose by *Acetobacter xylinum*. Researchers used pineapple syrup, a food processing waste, as low cost substrate for the production of lactic acid using *Lactobacillus lactis* and enzyme invertase to hydrolyze sucrose into glucose and fructose.[30,31] In line with these reports, *C. violaceum* UTM5 showed good adaptability in growing and producing violet pigment in LPW by utilizing the sugars, cations, anions and other organic substances naturally present in the medium. However, the pigment yield gradually decreased after concentration of 10 % (v/v) liquid pineapple waste. This suggested the possible role of the

additional carbon source in suppressing the production of violet pigment most probably via catabolite repression. A similar situation was reported by Aruldass *et al.*[23] where addition of lactose resulted in the inhibition of red pigment (prodigiosin) production.

Other types of agro-industrial based medium used by other researchers for the pigment production by microorganisms are summarized in Table 2. A different approach was performed by Ahmad *et al.*[16] using sugarcane bagasse immobilized *C. violaceum* in flow through column. L-tryptophan (100 mg L^{-1}) was pumped into the column to promote growth of bacteria and a yield of 150 mg L^{-1} pigment was obtained after 24 h. It was mentioned that lignocellulosic components in the sugar cane bagasse served as carbon source in pigment production. A red pigment, prodigiosin was produced by *S. marcescens* UTM1 using brown sugar and it was reported that presence of furfural derivatives in the medium enhanced the pigment production.[23] Korumilli and Mishra[32] reported the use of fruit waste extract (pineapple, orange and pomegranate) as a sole carbon source for *Rhodotorula rubra* in carotenoid pigment production. Many other parameters were optimized using one factor at a time and Box-Behnken design to obtain high yield of carotenoid.

This present study obtained highest yield of pigment among the other reported agro-industrial based medium as the volatile and aroma compounds present in liquid pineapple waste serves a better source of carbon and nitrogen for the bacterial growth and violet pigment production. Thus, suitable agro-industrial based medium are applicable for selected pigment production.

3.3 Effect of L-tryptophan on pigment production

From this study, the yield of pigment significantly increased as the concentration of L-tryptophan increased (Fig. 4). A maximum yield of 367 ± 2 mg L^{-1} (p<0.001) was achieved at 150 mg L^{-1} L-tryptophan and a slight decrease in the pigment yield was observed at 200 mg L^{-1} of L-tryptophan. Researchers reported that L-tryptophan act as a precursor and formed basic structure of violacein.[26] It was found that all the carbon, nitrogen and hydrogen atoms of violacein were derived from two molecules of L-tryptophan and the oxygen atoms are from oxygenation of indole rings of intermediate violacein compound. Initially, two L-tryptophan molecules (sole precursors) undergo condensation followed by several reaction steps in the pyrrolidone-containing scaffold of the final violacein pigment with the assist of genes,

namely *vio*A, *vio*B, *vio*C, *vio*D and *vio*E.[6,7,33] Tryptophan is oxidized by *vio*A gene (L-amino acid oxidase) to indole-3-pyruvic acid (IPA) imine. The gene *vio*B is responsible for the oxidative coupling of two molecules of IPA iminie to form pyrrole/intermediate dimeric core (X). However, the intermediate of dimeric structure (X) is unidentified. Compound X spontaneously undergo intramolecular condensation to form chromopyrrolic acid (CPA). Alternatively, the intermediate dimeric structure (X) undergoes1,2-shift of indole substituent by *vio*E to form protodeoxyviolaceinic acid. This path requires a subsequent four-electron oxidation to intsal the ketone of the pyrrolidone and generates protodeoxyviolaceinic acid/prodeoxyviolacein. The gene *vio*C in the presence of NADPH acts to hydroxylate 2-position of the right side indole ring of protodeoxyviolaceinic acid to form deoxyviolaceinic acid/deoxyviolacein. On the other hand, protodeoxyviolaceinic acid undergoes hydroxylation reaction by catalyzation of *vio*D with the presence of NADPH to form protoviolaceinic acid. Addition of *vio*C to this intermediate with NADPH successfully produced violaceinic acid/violacein. [6,7,33]

3.4 Violet pigment production in a 50 L bioreactor

C. violaceum UTM5 showed good adaptability to grow in the static to shaking (ST→SK) condition with the supplementation of L-tryptophan in 50 L bioreactor using liquid pineapple waste. A high yield of 16257 ± 440 mg L^{-1} was obtained after 24 h of cultivation in dark condition. The violet pigment was higher than that reported by Nakamura *et al.*[34] who evaluated the production of pigment (violacein) by a phychrotropic bacterium RT102 strain using a modified growth medium (containing glucose, casein, yeast extract, K_2HPO_4 and $MgSO_4$). A yield of 3700 mg L^{-1} was obtained from cultivation of the strain in a 3 L bioreactor at 20 °C, pH 6 for 30 h.

In this study, pigment yield was also found to be four times higher than that reported by Yang *et al.*[35] who cultivated a recombinant *Citrobacter freundii* using a fed-batch approach. The bacterium was fermented in a 5 L bioreactor with 2 L of initial working volume at 20 °C using an automated agitation adjustment between 100 and 800 rpm for 50 h. Glycerol, NH_4Cl and L-tryptophan were fed to enhance the pigment production and 4130 mg L^{-1} pigment was produced. As compared to the approaches that were reported for violet pigment production, the present study showed highest yield of the violet pigment when the *C. violaceum* UTM5 was grown in LPW in a 50 L bioreactor.

In Fig. 5, violet pigment production increased proportionally with the cell turbidity and cell density of *C. violaceum* UTM5 in a 50 L bioreactor. It is known that microorganisms produced secondary metabolites during stationary phase as the nutrients needed for respiration depleted as cells grow.[36] A different scenario was observed in this study as the violet pigment was observed at the early fermentation stage of *C. violaceum* UTM5. This is mainly due to the presence of violet pigment in the LPW medium at the initial stage of fermentation, which was transferred from the violet pigmented seed cultures. It was reported that violacein protect the *C. violaceum* membrane from oxidation or perioxidation.[37] This indicates that growth of *C. violaceum* UTM5 is not affected by violet pigment production. Higher pigment production was observed at later growth stage of *C. violaceum* UTM5, where there was high cell density is suggested to due to the control of quorum sensing system.[38,39] In quorum sensing, a bacterial cell able to sense the cell density by the accumulation of signalling molecules. The exchange of signalling molecules is essential in the coordination of gene expression in *C. violaceum* population and may regulate the violet pigment biosynthesis.

In this study, a simple extraction technique was employed as the violet pigment was secreted extracellularly in LPW at the surface with a fragile pellicle.[40] Thus, cell disruption and large amounts of solvent were not needed in extracting the violet pigment from *C. violaceum* UTM5. During fermentation in LPW, the *C. violaceum* UTM5 achieved slight increase in specific growth rate (μ) and pigment production (μ_p) of 0.164 h^{-1} and 0.161 h^{-1}, respectively as compared to fermentation in NB, which reached a rate of 0.154 h^{-1} (μ) and 0.138 h^{-1} (μ_p). The growth rate and pigment production rate in LPW was 1.1 and 1.2 fold higher as compared to NB respectively. This result obtained in present study suggest that LPW with additional L-tryptophan supplementation provides adequate amount of organic carbon and nitrogen compounds which are essential for satisfactory cell growth and pigment production.

It is difficult to estimate the market for bacterial pigments produced by bioprocesses, due to either lack of statistics of regional, low-technology products namely annatto extracts or the production is discontinued over many small companies worldwide.[41] However, growing priorities for bacterial pigments in textile dyeing, pharmaceuticals and cosmetics may increase its demand in industries. Natural pigments may be several times more expensive than synthetic pigments, in some cases. In the present study, the use of LPW for violet

pigment production reduced the production cost from 281.20 USD in NB to 235.70 USD in LPW. The production cost was also reduced by using a simple pigment extraction technique in extracting violet pigment from *C. violaceum* UTM5. Moreover, the utilization of cheap and renewable substrate, i.e. LPW as growth medium for *C. violaceum* UTM5 will make the price of pigments competitive with synthetic pigments. Although there are several challenges associated with scaling up of pigment production, present study has successfully overcome these challenges by producing violet pigment in LPW from *C. violaceum* UTM5 on a large scale. This eventually provides a potential route for reintroducing bacterial pigments to a cost-sensitive world.

The effluent from fermentation process was treated upon aeration for three days and the COD value decreased significantly by 75 % from an initial value of 85533.3 ± 2600.6 mg L^{-1} to 21183.3 ± 256.6 mg L^{-1} (p<0.001; Table 3). However, the rate of turbidity (252 ± 5.6 NTU) and total solid suspended (TSS; 214.3 ± 2.5 mg L^{-1}) values increased to 844.3±15.6 (p<0.001) NTU and 686.3 ± 17.8 (p<0.001) mg L^{-1}, respectively. Treated effluent was then passed to coagulation tank for second stage of treatment using PAC-Al_{13} to remove pollutants such as organic matters. Reduction of 92 % COD and odour was observed after the coagulation with PAC-Al_{13} units. Turbidity and TSS values were observed after coagulation step to 27.7 ± 2.5 (p<0.001) NTU and 23.7±2.1 mg L^{-1} (p<0.001), respectively. At the final stage of treatment, treated effluent was filtered through multi media filter filled with slow sand, carbon filter filled with granular activated carbon and 0.2/0.4 micron membrane filter before disposing into the water. A complete significant COD reduction of 99 % (1 mg L^{-1}; p<0.001) was achieved at the final stage of treatment, which met the discharge limit of Malaysian Standard B.[18] A maximum removal percentage of turbidity and TSS of 99 % was achieved after the final stage of treatment. Removal of turbidity is essential for an effective disinfection process. It was reported that in the water environment, the turbidity agent can protect the viral and bacterial organism against the disinfectant matter.[42] The effluent treated in this work recorded the final concentration of COD, turbidity and TSS of 1 mg L^{-1}, 1.65 NTU and 2.5 mg L^{-1}, respectively. This clearly demonstrated that the present pilot scale treatment system successfully treated the fermentation effluent and was safe to be disposed into the nearby water system.

3.5 Stability of violet pigment

Studies on violet pigment stability to pH were carried out and the colour was stable from pH 1 to 11 (Fig. 6a). On the other hand, difference in absorbance values at 575 nm was observed as colour changed from violet to pale violet when the pH of the solution increased to pH 14 (Fig. 6a). This is due to the destruction of electronic pi system in violet pigment structure which is responsible for colour change at higher pH.[43] It has been reported that violacein exhibit a colour change from darker blue at pH 2 to green at pH 13. In alkaline condition, excess OH^- ions from NaOH deprotonates the phenolic group of the hyroxyindol and amine group of oxoindol and pyrrolidone causing the formation of anion and destruction in the conjugated structure of violet pigment.[16] This was supported from the hue angle values where the range is from 280-290° (violet colour) for pH 1 to 11 and 190.20° (pale violet) for pH 14.

Violet pigment discoloration in the presence of light was higher than in the dark condition as the absorbance values decreased over a period of time (Fig. 6b). At 0 day, the hue angle values of pigment were 288 ° for both conditions and the values decreased to 262 ° and 286 ° for light and dark conditions at 28 days of storage, respectively. This result demonstrates that violet pigment is sensitive to illumination. The absorption of the light in the UV and visible ranges leads to the excitation of electron chromophore group to unstable and short-lived excited state. Higher reactivity of violacein molecule towards undesireable chemical reaction such as photo-oxdation may cause by excess energy trapped in the excited molecules.[16,43] The eventually cause the decrease in colour intensity of the violet pigment under light condition. Hence, violet pigment was stable in the absence of light.

Similarly, discoloration of violet pigment was observed at 60 and 100 °C. Violet pigment found to be stable at 25 and 30 °C upon storage for 28 days as compared to 100 °C. The pigment colour changed from violet to pale violet upon storage at 100 °C and absorbance of pigment decreased as the temperature rise to 100 °C (Fig. 6c). The values of hue angle were 289 ° (25 °C), 288 ° (30 °C), 287 °(60 °C) and 287 ° (100 °C) at 0 day and the values decreased to 285 ° (25 °C), 283 °(30 °C), 278 ° (60 °C) and 204 ° (100 °C) at 28 day of storage. This shows that violet pigment is sensitive to higher temperatures. The colour degradation observed in this study is a common characteristic of natural pigments, which usually compensated by proper pigment dosage.[9]

3.6 Antimicrobial activity

Infectious diseases continue to ravage the human population, and they account for approximately half of the mortality rates in tropical countries in this 21st century. However, spreading of multi-drug-resistant bacteria has severely reduced the efficacy of antibacterial agents, thus increasing therapeutic failures.[44] Indication of their devastating nature becomes an alarming statistics. Since there were no reports on antimicrobial activities of violet pigment produced in liquid pineapple waste from local isolate, *C. violacein*, it offers an attractive choice in exploring as a promising antimicrobial agent.

The antimicrobial potency of violet bacterial pigment was quantitatively determined by the microdiluton method. Minimum inhibition concentration (MIC) values exerted by violet pigment are presented in Table 4. The pigment showed considerable antimicrobial activity against tested strains with MIC values ranging from 7.8-1000 μg mL^{-1}. DMSO control solution showed no inhibitory effect at 12.5 % and lower for *S. aureus* ATCC 29213, MRSA ATCC 43300 and *P. aeruginosa* ATCC 27853. As for *K. pneumoniae* ATCC 700603 and *C. albicans* ATCC 10231 strains, DMSO control solution showed no inhibitory effect at 6.25 % and lower. The present study showed that crude violet pigment does not possessed antifungal activity because it is inactive against *C. albicans* ATCC 10231. It has MIC values of 125 μg $mL^{-1}$and 1000 μg mL^{-1} against *P. aeruginosa* ATCC 27853 and *K. pneumonia* ATCC 700603, respectively. Of interest, violet pigment was most active against *S. aureus* ATCC 29213 and MRSA ATCC 43300 with MIC values of 7.8 μg $mL^{-1}$and 15.6 μg mL^{-1}. The violet pigment differs in its antibacterial potency against selective strains. The MBC values obtained for violet pigment and standard antibiotics are higher than their MIC values (Table 5). Since the calculated ratios of MBC/MIC obtained were above 1, the pigment and antibiotics possessed bacteriostatic activity against selected strains. Thus, the crude violet pigment and antibiotics were only able to inhibit the growth rather than kill the selected strains.

Antimicrobial activities of violet pigment from psychrotropic bacterium RT102 strain on various microorganism strains were also reported previously by Nakamura *et al*.[34] However, the MIC value for *S. aureus* ATCC 29213 in the present study is found to be lower that reported value. It was reported that high concentration of violet pigment (above 15 μg mL^{-1}) able to kill the microorganism cells. The pigment inhibited *Bacillus lichemiformis*,

Bacillus megaterium, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Flavobacterium balustinum* with MIC values of 15 μg mL^{-1}. Martins *et al.*[45] carried out a different approach by loading the violacein with polymeric poly-(D,L-lactide-co-glycolide) nanoparticles and testing its antibacterial activity. The nanoparticels-loaded violacein were at least three times more effective than free violacein against the *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 strains with the MIC value of 2 μg/mL. This may be due to the activeness of nanoparticles loaded violacein upon internalized in the cells in a more efficient way than in the free form of violacein. It is also reported that *S. aureus* ATCC 29213, similar strain with the present study could be inhibited by free violacein at a MIC value of 5.1 μg/mL, which is lower than the current MIC value. This indicates the effectiveness of violacein inhibiting the growth of the bacteria upon purification from the crude violet pigment.

In the present study, the crude violet pigment was active against *S. aureus* ATCC 29213 and MRSA (ATCC 43300) being 5 and 10 times less potent than the standard antibiotic, vancomycin, respectively. The pyrrole N-H structures present in **(1)** and **(2)** may contribute to the antibacterial activity of crude violet pigment against selective strains. Similar role of pyrrole moieties as antibacterial agents was also reported by Marchal *et al.*[46] as pyrrolic N-H moieties of prodigiosene were active against Gram-positive bacteria.

3.7 Cytotoxicity

The cytotoxicity of violet pigment was determined using an *in vitro* assay with the Chinese hamster lung (V79-4) cells. The pigment was relatively toxic with LC_{50} value of 3.78 ± 0.03 μg mL^{-1}. Upon calculating the selectivity index of violet pigment against the pathogens by dividing the LC_{50} by the MIC, values between 0.00 and 0.485 were obtained, indicating that the violet pigment is much more toxic to the V79-4 cells than to the pathogens (Table 6). The pigment had the best SI value of 0.485 against *S. aureus* ATCC 29213, but for other pathogens, the SI values were below 0. It is considered that the ratio for a good selectivity or therapeutic index for a remedy or drug should be >10, which is a cut-off point ensuring the overdose does not put the life of the patient in danger.[47] In this study, low selectivity indexes (SI < 1) indicated that bioactivity of violet pigment was most likely owing to general its toxic effects[20] A standard cell-based toxicity assays are needed to be performed *in vitro* at an early stage of the drug development process in order to ascertain the likely safety of the bacterial pigment for their potential use.[48] Thus, it is possible that isolation of bioactive compounds

and chemical modification experiments could reduce the toxicity and enhance bioactivity of the pigment to increase their potential usefulness in future.[20]

3.8 Characterization of violacein and deoxyviolacein

In FT-IR analysis for **(1)**, strong absorption band at ν_{max} 3421 cm^{-1} (NH), 1669 cm^{-1} and 1689 cm^{-1} (carbonyl amide, NH-C=O), 1621 cm^{-1} (olefin, C=C) and 1279 cm^{-1} (amine, C-N). Broad absorption of OH was evident at ν_{max} 3237 cm^{-1}. Although similar absorptions were detected for **(2)**, absorption for OH was not detected. These indicate that both compounds' pattern is similar to that reported by Wille and Steglich.[49] The position of each of the proton in the **(1)** and **(2)** structure are indicated on each ^{1}H-NMR spectra shown in Fig. 7a and Fig.7b, respectively. In the ^{1}H-NMR spectrum (Fig. 7a), a chemical shift of the hydroxyl group in **(1)** exhibited δ 9.35 ppm as a singlet, which can be found in the indole skeleton of **(1)** but similar chemical shift was not detected for **(2)** compound (Fig. 7b). In addition, three distinct signals corresponding to NH protons were detected for **(2)** compound.

Two doublet signals at δ 10.75 ppm (NH˝), 11.90 ppm (NH′) and a singlet signal at δ 10.63 ppm were assigned to isatin, indole and lactam skeleton, respectively (Fig 7a). Multiplet signal was detected at δ 7.31 ppm (H-5′ and H-6′) corresponding to two protons in indole skeleton of **(2)** (Fig. 7b). It was found that **(1)** has 9 quaternary carbons and a strong signal at δ 153.4 ppm (C-5′-OH) was detected in **(1)** indicates the presence of hydroxyl residue at the C-5′. However, similar signal for hydroxyl residue was not detected for **(2)**. Two carbonyl carbon were detected at δ 170.7 and 172.1 ppm (C=O) for **(1)** and δ 170.6 and 171.9 ppm (C=O) for **(2)**. These indicate that both compounds' chemical shifts are similar to that reported by others.[49,50]

4 Conclusion

This study demonstrated the potential application of liquid pineapple waste supplemented with L-tryptophan as an alternative growth medium for the production of violet pigment by *C. violaceum* UTM5. Effluent from the fermentation was successfully treated using aeration, coagulation and filtration techniques for eco-friendly disposal. Violet pigment can achieve better stability during the storage of pH 7, temperature 25 – 30 °C and to be in dark for a month. The violacein and deoxyviolacein compounds were isolated from the violet pigment and confirmed by FT-IR, NMR and LC-MS analyses. Crude violet pigment showed

antibacterial activity, but has residual toxicity against the Chinese hamster lung cells. However, isolation of the active compound and synthesizing the analogues of violacein may exhibit an interesting therapeutic window for potential use as antibiotics. The use of low cost and easily available agricultural waste (liquid pineapple waste) in place of more expensive conventional complex medium should expedite large-scale production of this bacterial secondary metabolite. The findings are certainly encouraging to develop a cost effective natural colorant that would be of more attraction to product developers namely pharmaceutical industries.

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Table 1. Pigment production in different culture conditions

Medium	Pigment concentration (mg L^{-1})			
	Culture condition			
	ST→ST	**ST→SK**	**SK→ST**	**SK→SK**
NB	170 ± 9[a]	258 ± 8[c]	178 ± 2[b]	195 ± 5

NB means nutrient broth, ST means static and SK means shaking condition. Values for yield of pigment are presented as mean±standard deviation from experiments in triplicate [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ compared to SK→SK condition (t-test)

Table 2. Comparison analysis of pigment production by microorganisms in different low cost medium

Substrate	Pigment	Strain	Yield (mg L^{-1})	Reference
Sugar cane bagasse	Violet/violacein	*Chromobacterium violaceum*	150	15
Brown sugar	Red/prodigiosin	*Serratia marcescens*	237	17
Fruit waste	Orange/carotenoid	*Rhodotorula rubra*	2.98	31
Liquid pineapple waste	Violet/violacein	*Chromobacterium violaceum* UTM5	285	Present study

Table 3. Profile for the removal of pollutants using the pilot scale effluent treatment system

	Initial concentration	**After aeration**	**After coagulation**	**After filtration**	**Percentage of removal (%)**
COD (mg L^{-1})	85533.3 ± 2600.6	21183.3 ± 256.6[c]	6233.3 ± 202.1[c]	1[c]	99
Turbidity (NTU)	252 ± 5.6	844.3 ± 15.6[c]	27.7 ± 2.5[c]	1.57 ± 0.2[c]	99
TSS (mg L^{-1})	214.3 ± 2.5	686.3 ± 17.8[c]	23.7 ± 2.1[c]	2.7 ± 0.6[c]	99
pH	4.6	5.3	5.09	7.2	-

Values for concentrations are presented as mean±standard deviation from experiments in triplicates [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ compared to initial concentration (t-test)

Table 4. Minimum inhibition concentration (MIC) of violet pigments and standard antibiotics

Strain	Crude Violet Pigment (µg mL^{-1})	Antibiotics (µg mL^{-1})			DMSO not inhibiting (%)
		VC	GM	AM B	
S. aureus ATCC 29213	7.8	1.56	-	-	12.5
MRSA ATCC 43300	15.6	1.56	-	-	12.5
P. aeruginosa ATCC 27853	125	-	0.78	-	12.5
K. pneumoniae ATCC 700603	1000	-	0.78	-	6.25
C. albicans ATCC 10231	-	-	-	25	6.25

VC, Vancomycin; GM, Gentamycin; AM B, Amphotericin B

Table 5. Minimum bactericidal/bacteriostatic concentration of violet pigment and standard antibiotics

Strain	Crude Violet pigment (µg mL^{-1})	Antibiotics (µg mL^{-1})	
		VC	GM
S. aureus ATCC 29213	62.5 (Bacteriostatic)	6.25 (Bacteriostatic)	-
MRSA ATCC 43300	125 (Bacteriostatic)	6.25 (Bacteriostatic)	-
P. aeruginosa ATCC 27853	500 (Bacteriostatic)	-	3.125 (Bacteriostatic)

VC, Vancomycin; GM, Gentamycin

Table 6. Selectivity index (SI) values of violet pigment against selected bacterial strains

Strain	Selectivity Index (SI) (LC_{50} V79-4=3.78 ± 0.03 µg mL^{-1})
S. aureus ATCC 29213	0.485
MRSA ATCC 43300	0.243
P. aeruginosa ATCC 27853	0.030
K. pneumoniae ATCC 700603	0.004

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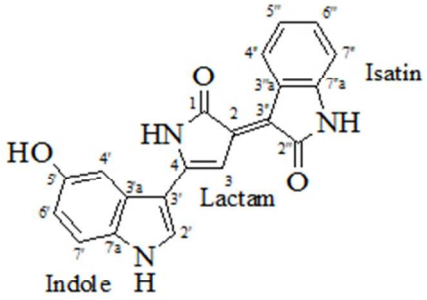
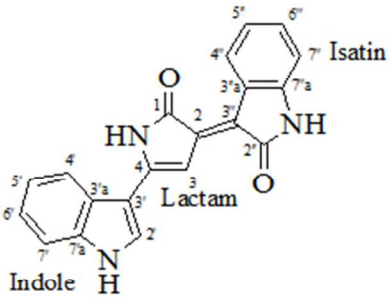
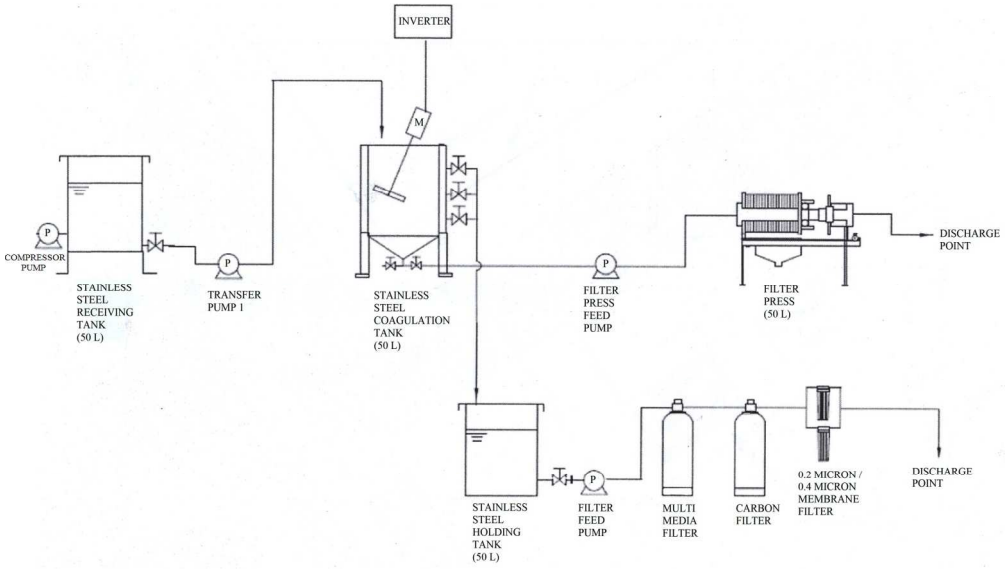


(1)

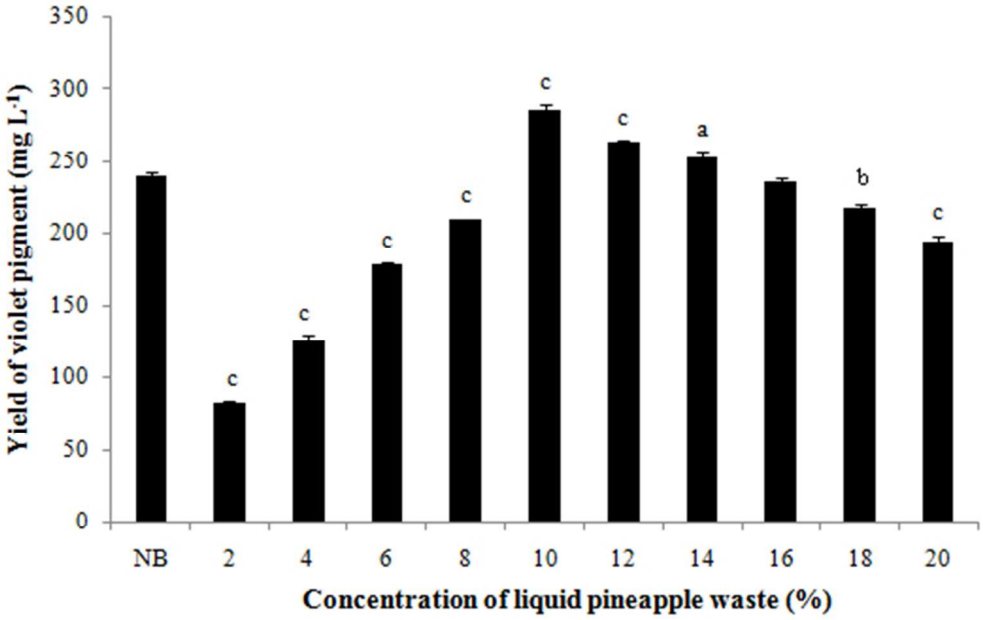


(2)

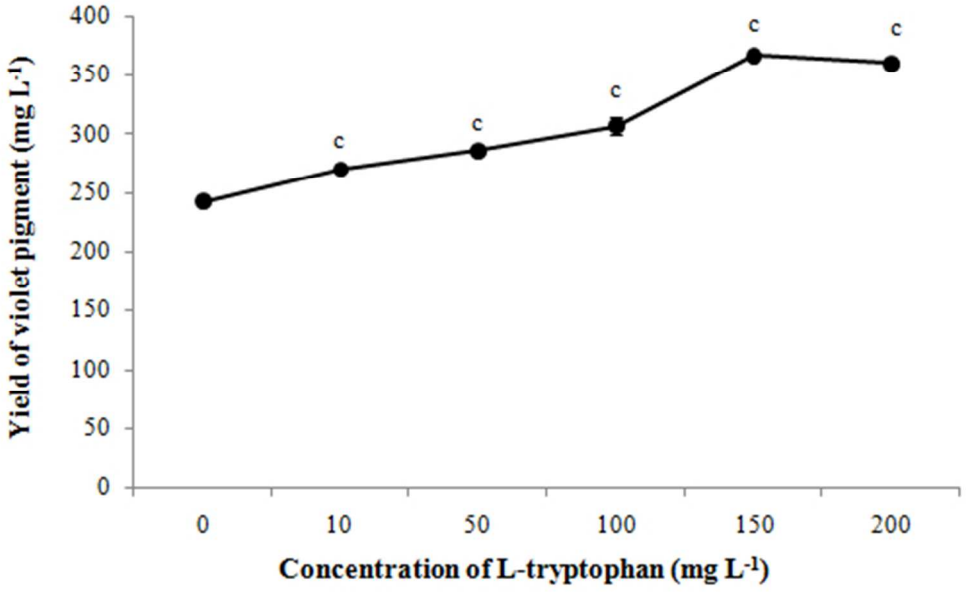
160x64mm (96 x 96 DPI)



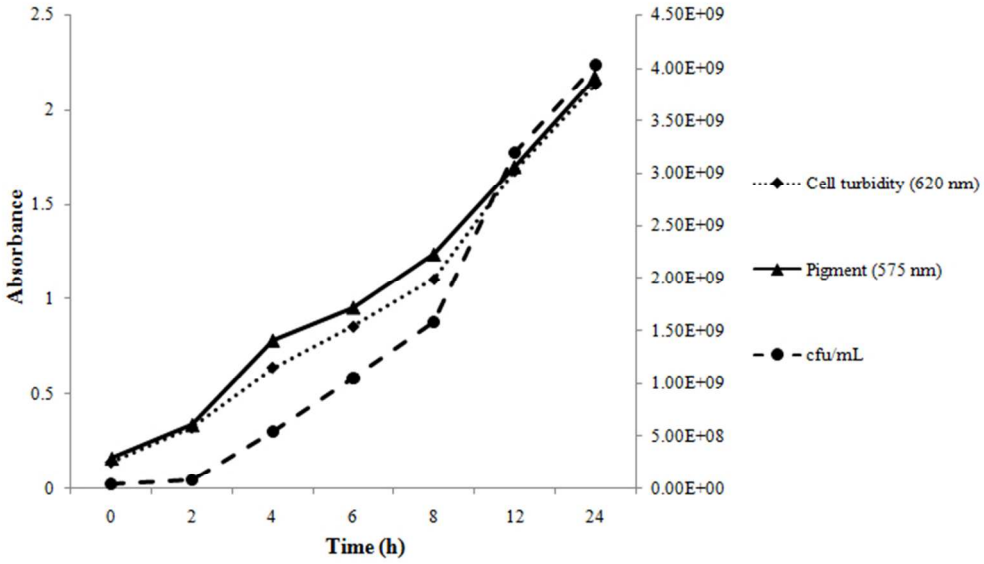
551x311mm (96 x 96 DPI)



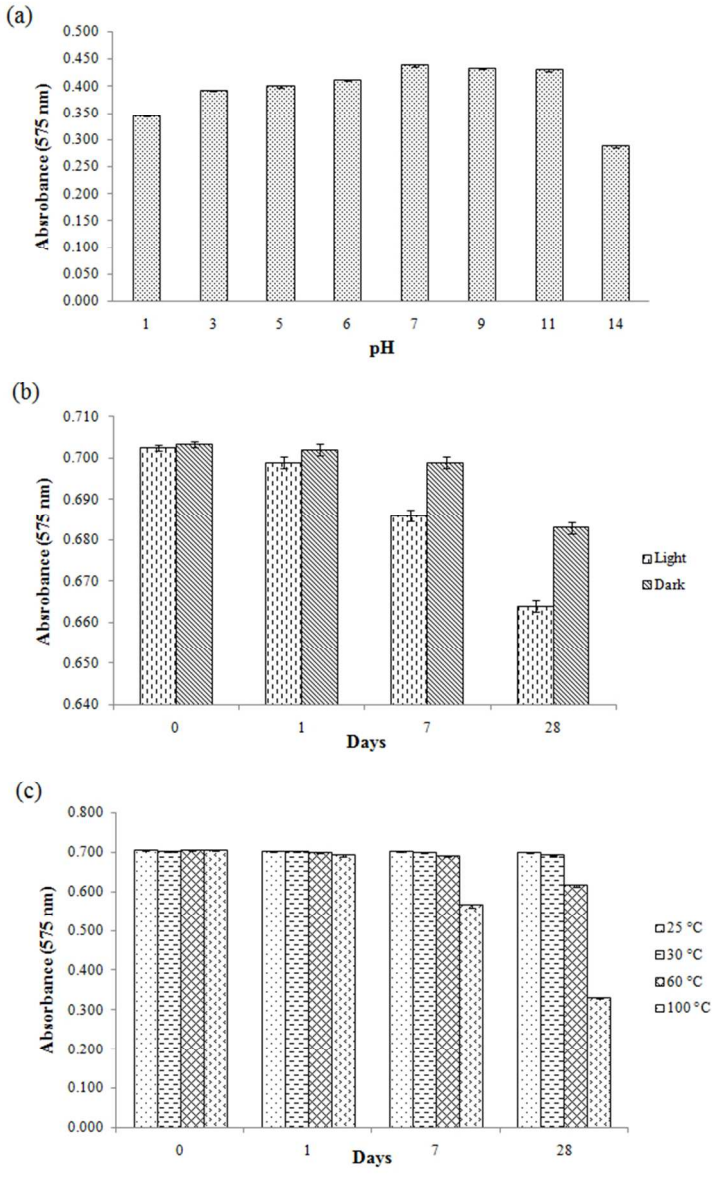
140x91mm (96 x 96 DPI)



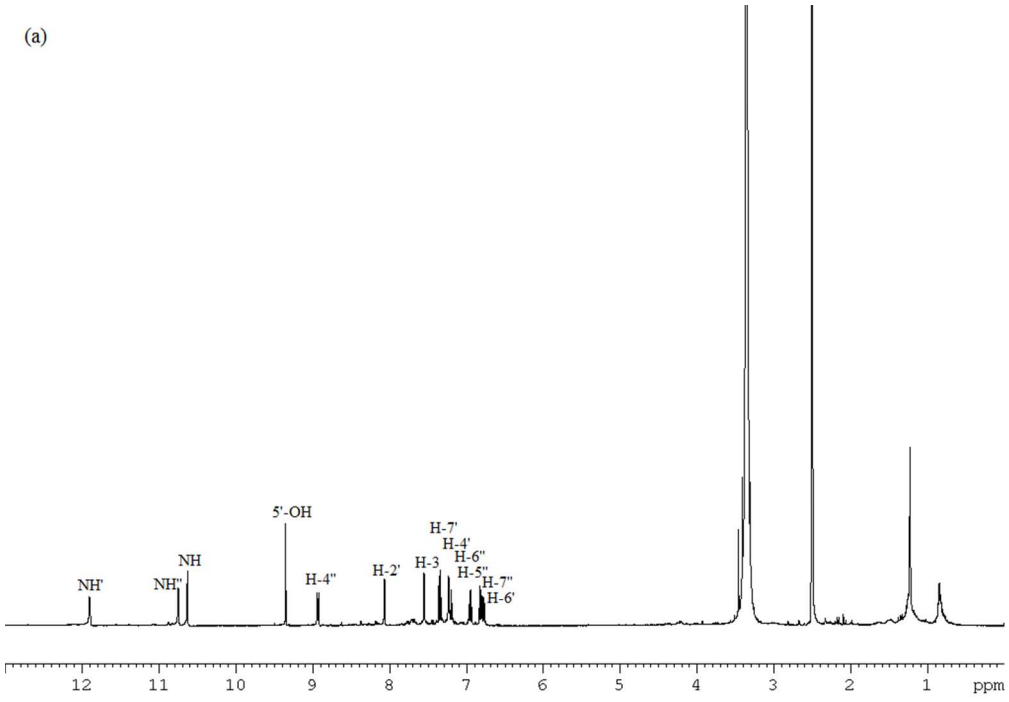
135x86mm (96 x 96 DPI)



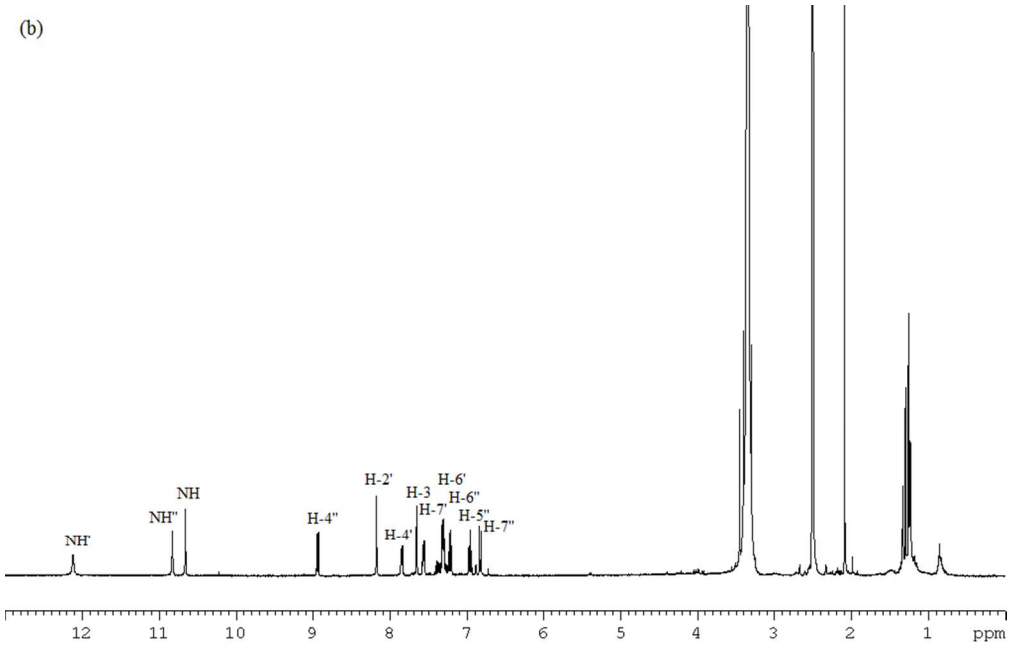
189x109mm (96 x 96 DPI)



176x273mm (96 x 96 DPI)



268x185mm (96 x 96 DPI)



267x171mm (96 x 96 DPI)