

Chapter 2

Isolation of Pigment-Producing Bacteria and Characterization of the Extracted Pigments

Abstract Bacteria produce pigments for various reasons and it plays an important role. Some bacteria such as cyanobacteria have phycobilin pigments to carry out photosynthesis. Other example for pigment-producing bacterial strains includes *Serratia marcescens* that produces prodigiosin, *Streptomyces coelicolor* (prodigiosin and actinorhodin), *Chromobacterium violaceum* (violacein) and *Thiobacillus versutus* (natronochrome and chloronatronochrome). These bacteria can be isolated/cultured/purified from various environmental sources such as water bodies, soil, on plant, in insects and in man or animal. Various growth mediums can be used to isolate different types of bacteria. However, due to the high cost of using synthetic medium, there is a need to develop new low cost process for the production of pigments as well as during the isolation procedure. The use of agro-industrial residues for example, would provide a profitable means of reducing substrate cost. Pigment produced by the bacteria can be isolated using solvent extraction. These pigments can be further purified and characterized for physical and chemical characteristics using various instrumental-based analytical techniques such as TLC, UV-vis Spectroscopy, FTIR, ESI-MS, NMR HPLC and Gel Permeation Chromatography.

Keywords Pigment • Bacteria • Isolation • Characterization • NMR, HPLC • Extraction • Medium

2.1 Growth Medium

Nutrient broth (8 g L⁻¹, Merck) and Nutrient agar (20 g L⁻¹, Merck) were used as growth medium. The mediums were sterilized by autoclaving at 121 °C, 103.42 kPa for 15 min where the agar was allowed to harden followed by incubation for 24 h at 30 °C to ensure that it was free from contamination.

Table 2.1 Characteristics of water and soil samples collected from Brackishwater Aquaculture Research Centre, Johor

Sample	Sampling location/Type of sand	color	°C	pH
W1	Recycle water tank (<i>siakap</i> sp.)	Greenish Brown	29.7	6.24
W2	Recycle water tank (<i>tilapia</i> sp.)	Cloudy grey	31.9	6.24
W3	<i>Rotifer</i> breeding tank	Clear	28.3	6.22
W4	Fish breeding tank	Clear	32.1	6.29
W5	Water point source (from sea)	Cloudy grey	32.4	6.21
W6	Shrimp pond	Clear	28.3	6.14
W7	Organic waste tank (red <i>tilapia</i> sp.)	Cloudy grey	29.5	6.24
W8	Organic waste tank	Cloudy grey	28.5	6.18
S1	Clay	Orange	n/a	7.12
S2	Small sand	Grey	n/a	6.02
S3	Small sand	Grey	n/a	6.11
S4	Clay	Yellow	n/a	5.57
S5	Mixture of sand and clay	Dark green	n/a	6.23
S6	Clay	Orange	n/a	6.01
S7	Small sand	Grey	n/a	7.11
S8	Clay	Orange	n/a	6.14
S9	Mixture of sand and clay	Dark green	n/a	4.93
S10	Clay	Orange	n/a	6.22
S11	Small sand	Black	n/a	5.24
S12	Mixture of sand and clay	Dark green	n/a	6.11
S13	Small sand	Black	n/a	5.77
S14	Mixture of sand and clay	Dark grey	n/a	6.34
S15	Small sand	Grey	n/a	6.65
S16	Small sand	Black	n/a	5.88

2.2 Location and Techniques of Sampling

Liquid and soil samples were obtained aseptically from the Brackishwater Aquaculture Research Centre (BARC), Johor and one oil refinery facility in Port Dickson, Negeri Sembilan. A total of 16 soil samples, consisting of a mixture of clay and sand, were collected in the vicinity of the wastewater treatment pond of the oil refinery while at the Brackishwater Aquaculture Research Centre, solid samples were collected near the shrimp pond and the liquid samples, from the effluent fraction of various tanks namely fish rearing, rotifer breeding, fish breeding and organic waste collector. All sampling procedures were carried out according to Standard Methods for the Examination of Water and Wastewater (Eaton and Franson 2005). Pre-sterilized 250 mL Schott bottles were filled with liquid and soil samples and ample air space (for liquid samples), about 2.5 cm, were left to facilitate mixing, aeration and thermal expansion normally encountered during handling and transportation. The bottles were placed inside an iced-box polystyrene container during the 2–3 h transportation journey to the laboratory (to minimize indigenous microbial activity and preserve original speciation of chemicals present). Some characteristics of the water and soil samples are shown in Table 2.1.

Table 2.2 Phenotypic characteristics for some of the bacterial colonies isolated from soil and water samples obtained from BARC, Johor and one oil refinery in Port Dickson, Negeri Sembilan

Bacteria ID	Colony appearance	Colony Characteristics		
		Form	Elevation	Margin
W5a	Light-Yellow	Irregular	Flat	Erose
S6a	Pale-Yellow	Punctiform	Convex	Entire
S7a	Yellow	Punctiform	Convex	Entire
S8a	Light-yellow	Filamentous	Raised	Fillamentous
S8b	Yellow-orange	Punctiform	Convex	Entire
S8c	Cream-yellow	Circular	Convex	Entire
S8d	Yellow-orange	Punctiform	Convex	Entire
S10a	Yellow	Punctiform	Convex	Entire
S10c	Cream-yellow	Punctiform	Convex	Entire
S1a	Violet	Punctiform	Raised	Entire
S1b	Opaque-yellow	Circular	Umbonate	Entire
S2d	Light-yellow	Punctiform	Raised	Entire
S5e	Pale-yellow	Punctiform	Raised	Entire
S6c	Pale-yellow	Punctiform	Umbonate	Undulate
S7a	Yellow	Punctiform	Convex	Entire
S10a	Light-yellow	Circular	Raised	Entire
S11a	Opaque red	Circular	Convex	Entire

2.3 Cultivation and Isolation of Cultures

Each of the liquid samples (2.5 mL) was aseptically transferred into a series of 250 mL Erlenmeyer flasks containing 22.5 mL NB medium followed by incubation at 30 °C, 200 rpm for 24 h (Certomat-B, B-Braun). One loopful of bacterial cultures was then transferred onto NA plates and incubated for 24 h at 30 °C for 24 h (Memmert, USA). Serial sub-culturings were carried out until single bacterial colonies were obtained. Similar experimental procedures were repeated for the soil samples using one gram of soil sample as inoculant. Single bacterial colonies were identified via the 16S rRNA gene sequencing analysis carried out by Vivantis Technology Sdn. Bhd., Malaysia. A total of 77 bacterial colonies (Table 2.2) were isolated from solid and liquid samples from the BARC, Johor (45 colonies) and oil refinery wastewater treatment plant at Port Dickson, Negeri Sembilan (32 colonies). Of the 45 colonies isolated from the BARC compound, isolate S8b was chosen for further studies based on its intense yellow-orange coloration when grown in NA and NB mediums (Fig. 2.1a,b). For bacterial colonies isolated from the oil refinery in Port Dickson, 8 were colored. Of these, isolate S1a that displayed gradual color change from grey to dark violet throughout incubation period, was chosen for further study (Fig. 2.1c, d).

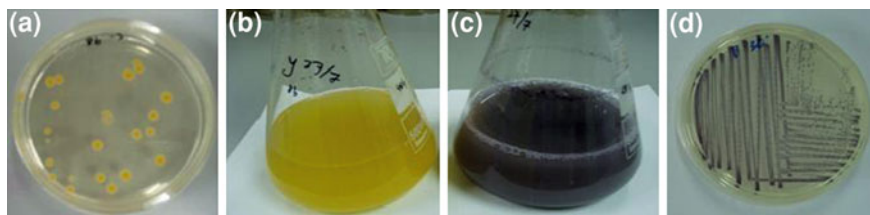


Fig. 2.1 Morphology of isolate S8b on (a) NA plate (b) NB medium; isolate S1a on (c) NB medium (d) NA plate

2.4 Characterization of Microorganisms (violet, yellow, red)

The characterization of bacteria was carried out by Vivantis Technologies Sdn. Bhd., Malaysia, using 16S rRNA sequence analysis. From the analysis, the pigment-producing bacteria were identified as follows; yellow–orange as *Chryseobacterium* sp., violet pigment as *Chromobacterium violaceum* (*C. violaceum*) and red pigment as *Serratia marcescens* (*S. marcescens*).

C. violaceum, a Gram-negative bacteria belonging to the Rhizobiaceae family, is a saprophyte found in soil and water in tropical and subtropical areas. In most cases, it is a minor component of the total microflora (Balows et al. 1992). Its colonies are slightly convex, not gelatinous, regular and violets, although irregular variants and non-pigmented colonies can also be found (Sneath 1994). *C. violaceum* has been reported to produce a violet pigment called violacein. Violacein possesses anti-leishmanial (Leon et al. 2001), anti-viral (Andrighetti-Fröhner et al. 2003), antitumoral (Ueda et al. 1994, Melo et al. 2000) and anti-*Mycobacterium tuberculosis* (De Souza et al. 1999) activities. Other properties of *C. violaceum* include the production of cyanide (Michaels and Corpe 1965), the solubilization of gold (Faramarzi et al. 2004), the production of chitinolytic enzymes (Chernin et al. 1998), the synthesis of bioplastics (Steinbüchel et al. 1993) and environmental detoxification (Carepo et al. 2004).

The genus *Chryseobacterium* was created by Vandamme et al. (1994) to accommodate several species formerly classified in the genus *Flavobacterium*, i.e., *Chryseobacterium balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*. Six species, *Chryseobacterium defluvii* (Kämpfer et al. 2003), *C. joostei* (Hugo et al. 2003), *C. miricola* (Li et al. 2003), *C. daecheongense* (Kim et al. 2005), *C. formosense* (Young et al. 2005) and *C. taichungense* (Shen et al. 2005), have been added to the genus recently.

Chryseobacterium strains produce translucent colonies, shiny with entire edges, but on prolonged incubation the colonies were not visible as single entities probably due to the profuse production of extracellular substances. On NA, it can produce a bright yellow nondiffusible, nonfluorescent flexirubin pigment. It was also reported to have an ability to produce heat stable metalloproteases and protein deamidating enzymes (Venter 1987; Yamaguchi and Yokoe 2000).

2.5 Maintenance of Stock Culture

The LB-glycerol solution was used for long-term storage (months to years) of the bacterial stock culture. The bacteria were first inoculated onto NA plate and incubated at 30 °C for 24 h. The bacterial colonies formed were then transferred into LB-glycerol prior to storage at −20 °C. LB-glycerol was prepared using the following procedure; a mixture of tryptone (10 g), yeast extract (5 g) and NaCl (10 g) were dissolved in 1 L distilled water. pH of the solution was then adjusted to 7.0 using 0.1 M NaOH followed by autoclaving at 121 °C, 121 kPa for 15 min (HVE-50, Hirayama). The bacterial stock cultures were prepared by transferring 2 mL of active culture (from NA plates) into a series of 5 mL Bijou bottles containing 2 mL of glycerol, 25% (v/v) followed by storage at −20 °C prior to use. Another medium used for the maintenance of bacterial stock cultures was 0.1% (w/v) peptone water (Gillis and Logan 2005), which was used specifically for *C. violaceum*. This was due to the inability to revive its growth in broth medium upon storage for 14 days in LB-glycerol. The presence of tryptophan-rich peptic digest of animal tissue in dilute peptone water (MacFaddin 1980) allows the survival of microorganisms up to several years (Gillis and Logan, 2005). Tryptophan is a precursor in the biosynthesis of violacein and its production is essential for pigment production in *C. violaceum* (Vasconcelos et al. 2003). The absence of essential precursors, cofactors and/or accessory proteins in the host organism would disrupt normal functions of various biosynthetic enzymes (August et al. 2000).

2.6 Characterization of Pigments

2.6.1 Extraction of Pigments

The bacterial cells were first grown for 24 h followed by centrifugation at 7500 rpm for 20 min (SIGMA 4 K-15, B.Braun). Both the supernatant and bacterial cell pellets were extracted (7500 rpm, 20 min) using either 95% (v/v) methanol (*S. marcescens* and *C. violaceum*) or 99.5% (v/v) acetone (*Chryseobacterium* sp.) in the ratio of 1: 5 (supernatant) or until the pellet was colorless, i.e., complete pigment extraction has been achieved. The bacterial cell pellet was then discarded while the supernatant was first extracted using ethyl acetate followed by concentration using rotary evaporator (BÜCHI R-210, Switzerland) at 50 °C with chiller temperature set at below 10 °C. The pigment concentration process was carried out until around 1% (v/v) of the initial solvent volume was left in the evaporation flask. The concentrated pigment was then transferred onto glass Petri dishes prior to drying for 3 days at 60 °C.

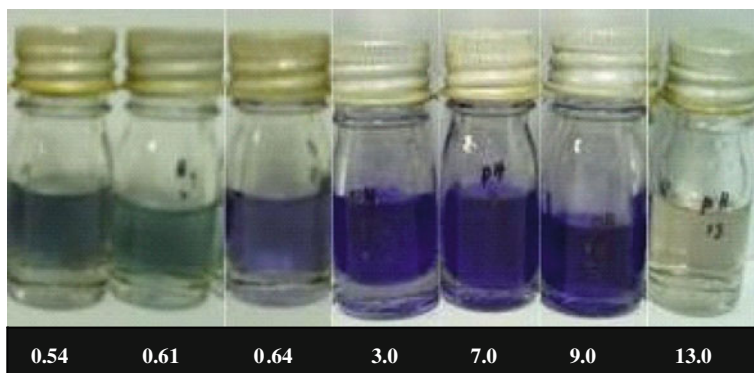


Fig. 2.2 The stability of crude-violet pigment at varying pH

2.6.2 Characterization of Crude-Violet Pigment

UV/vis and FTIR analysis

The purple pigment was tested for stability at various pH ranging from extremely acidic (0.54) to highly basic (13). At extremely low pH values, the purple pigment appeared greenish-blue, bright to dark-blue (3.0–9.0) while in highly basic condition, the pigment was almost decolorized (Fig. 2.2). The decrease in color intensity at high pH values can be attributed to the deprotonation of nitrogen by NaOH from the three conjugated rings at the pigment structure. This resulted in electron conjugations in the ring structure that gives it stability (Konzen et al. 2006). UV/vis analysis (200–800 nm scan; UV 1601PC; Shimadzu) of the crude-violet pigment (Fig. 2.3) showed maximum absorption peaks at 623.67 nm for pH 0.54, 576.61 nm (pH 0.61), 589.81 nm (pH 0.64) and 573.17 nm (pH 3, 7, 9 and 13). Methanol was used as blank.

The primary reason for stronger absorption of the pigment at longer wavelength (visible region) was the electron conjugation effect. A conjugated system requires lower energy for the electronic transition from the Π to Π^* orbitals. The presence of greater number of conjugated bonds resulted in λ_{max} appearing at the longer wavelength region (bathochromic shift) (Mohan 2007). Besides, the auxochromic or chromophoric substitution of five membered heteroaromatics ring present in the crude-violet pigment also causes a bathochromic shift and an increase in the intensity of the bands of the parent molecule. An auxochrome is an auxillary group which interacts with the chromophore resulting in bathochromic shift. The auxochromic group has the ability to provide additional opportunity for charge delocalization, thus providing smaller energy increments for transition to excited states. The charge delocalization by the contributing structures is in the presence and absence of electron donating-NHR group as shown in Fig. 2.4. From the figure, it is clearly shown that the charge delocalization is greatly enhanced by the presence of electron donating-NHR group. The added opportunity for stabilization

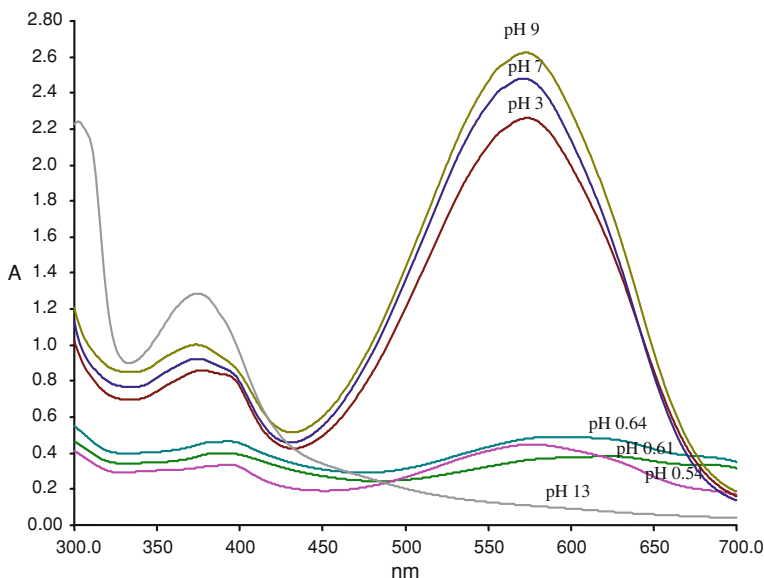


Fig. 2.3 UV/vis spectrum of crude-purple pigment at various pH values

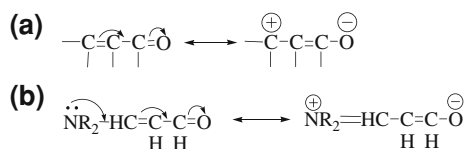


Fig. 2.4 Charge delocalization by the contributing structures in the (a) absence and (b) presence of electron donating-NHR group (Mohan 2007)

of the Π^* excited state brings the lowest excited state closer to the highest ground state and thus permits a lower energy, i.e., longer wavelength for transition (Mohan 2007).

FTIR analysis of the crude-violet pigment was carried out as follows; The RWS sample (1 mg) was ground with 200 mg of KBr (spectroscopic grade) in a mortar before pressed into 10 mm diameter disks under 6 tons of pressure. FTIR spectra were obtained on a FT-IR 8300, Shidmadzu spectrometer. The analysis conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 and $4,000 \text{ cm}^{-1}$. From the spectrum obtained (Fig. 2.5), crude methanolic extract of the violet pigment showed a broad band at $3,700\text{--}3,000 \text{ cm}^{-1}$ which corresponds to O–H stretching that also overlapped with N–H stretching of a secondary amide at 3256.53 cm^{-1} . Secondary amides are associated through H-bonding to form dimers (cis configuration) or polymers (trans configuration) resulting in the replacement of free N–H stretching band (Fig. 2.6). The weak band at 3256.53 cm^{-1} may be due to an overtone of the band at 1543.93 cm^{-1}

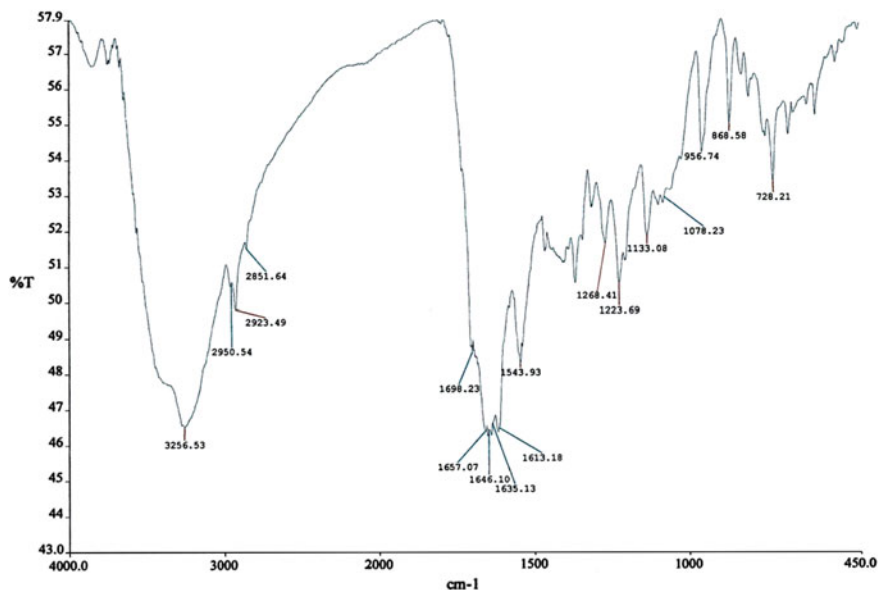


Fig. 2.5 FTIR spectrum for crude methanolic extract of violet pigment. (a) cis-dimer (b) trans-polymer

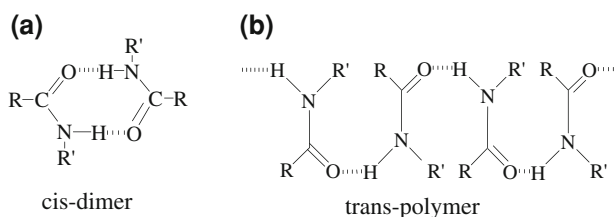


Fig. 2.6 Structure of secondary amides (a) cisoid–cisoid associations and (b) transoid–transoid polymeric association

(trans secondary amide) or combination of C=O stretching and N–H in-plane bending (cis secondary amide) (Mohan 2007).

The C=O stretching of this amide was depicted as doublet at 1657.07 cm^{-1} and 1635.13 cm^{-1} . This doublet is highly characteristic of the amide I band (predominantly C=O, 1635.13 cm^{-1}) and the amide II band (predominantly N–H in-plane bending, 1657.07 cm^{-1}). The frequency of C=O based amide is lower due to the conjugation of the carbonyl group with an aromatic ring. This results in the delocalization of the Π electrons of both unsaturated groups and reduces the double bond character of both bonds, hence reducing the typical carbonyl frequency from 1718 cm^{-1} to 1657.07 cm^{-1} and C=C stretching frequency from 1645 cm^{-1} to 1613.18 cm^{-1} . This situation can be explained further according to Fig. 2.7. In addition, the electron releasing groups (i.e., the amino group), tends to

Fig. 2.7 Resonance structure of carbonyl containing group in double bond structure (Mohan 2007)

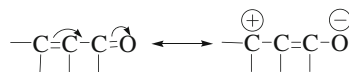


Table 2.3 $\nu_{\text{OH (free)}} \text{ cm}^{-1}$ and $\nu_{\text{C-O}} \text{ cm}^{-1}$ in alcohols and phenols

Nature of the hydroxyl compound	$\nu_{\text{OH (free)}} \text{ cm}^{-1}$	$\nu_{\text{C-O}} \text{ cm}^{-1}$
Primary alcohol	3640	1050
Secondary alcohol	3630	1100
Tertiary alcohol	3620	1150
Phenols	3610	1200

favor the polar contributing form by mesomeric effect, thus lowering the force constant of the C=O bond as well as decreasing the carbonyl-group stretching frequency. The strong C–O stretching vibration at 1223.69 cm^{-1} is highly characteristic of the C–O stretching vibration of phenol. In fact, further examination of the C–O stretching vibration is normally used to distinguish between primary, secondary and tertiary alcohols as shown in Table 2.3.

TLC, NMR and MS analysis

Crude methanolic extract of the violet pigment was spotted on a 0.20 mm pre-coated silica gel aluminum sheet (Merck Kieselgel 60F₂₅₄) and developed with a mixture of benzene : acetone (2 : 1) (Duran et al. 1994). The crude pigment showed a single spot with R_f value of 0.43 (vioacein) when viewed under UV light at 254 and 356 nm. Upon spraying with the vanillin-sulfuric acid mixture, a small sport with R_f value of 0.50 appeared which should represent deoxyviolacein, as suggested by DeMoss and Evans (1959). The vanillin-sulfuric acid mixture was prepared by mixing vanillin (0.5 g), methanol (80 mL), acetic acid (10 mL) and concentrated sulfuric acid (5 mL).

Powdered form of the crude extract was analyzed for ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) profiles using Bruker Avance 400 NMR spectrometer. Chemical shifts (in ppm) were reported relative to tetramethylsilane (TMS) with deuterated dimethyl sulfoxide (DMSO) as solvent. The ^1H NMR spectrum showed the presence of 13 protons (Fig. 2.8). Three singlet signals were observed at down-field region which corresponds to indole NH at δ 11.87 ppm, lactam NH (δ 10.72 ppm) and isatin NH (δ 10.60 ppm). Three aromatic protons in the indole skeleton which corresponds to the meta-couple and ortho-couple signals were detected at δ 6.79 ppm (H-7, dd, $J = 2.0 \text{ Hz}$, 8.8 Hz), δ 7.23 ppm (H-5, d, $J = 2.0 \text{ Hz}$) and δ 7.35 ppm (H-8, d, $J = 8.8 \text{ Hz}$). Other peaks were detected at δ 8.06 ppm (H-2, d, $J = 2.4 \text{ Hz}$) and δ 9.32 ppm (s, hydroxyl group). Chemical shifts observed at these lower-field regions were due to hydrogen bonding occurring between phenolic hydroxyl group with an ortho group (H-5 and H-7) that exists in the indole skeleton (Lambert and Mazzola 2004). Four protons were observed in aromatic isatin skeleton. These protons appeared at δ 8.93 ppm (H-19, d, $J = 7.6 \text{ Hz}$), δ 6.94 ppm (H-20, t, $J = 7.6 \text{ Hz}$), δ 7.20 ppm (H-21, t,

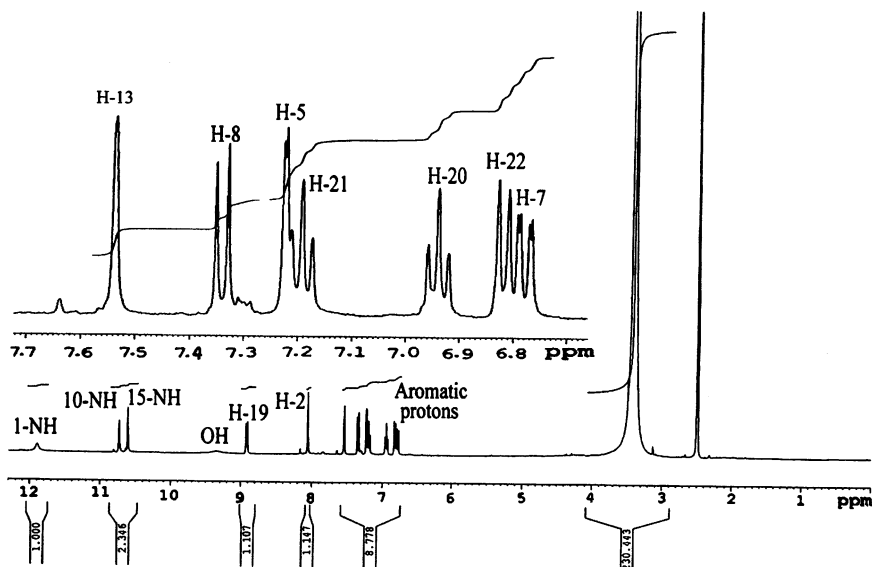
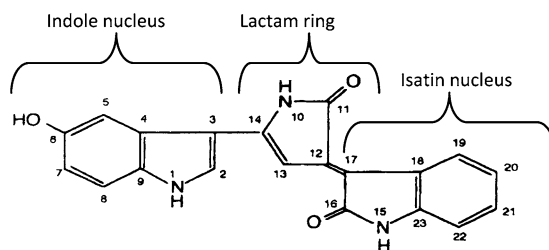


Fig. 2.8 ^1H NMR spectrum of violet pigment

Fig. 2.9 The structure of violacein



$J = 7.6$ Hz) and δ 6.83 ppm (H-22, d, $J = 7.6$ Hz). A doublet peak at δ 7.55 ppm with $J = 2.0$ Hz was assigned to H-13 in the lactam skeleton. The appearance of low-field signals from indole and pyrrole NH protons can be attributed to the presence of electron-withdrawing group substituents attached to the pyrrole ring. This corresponds to the hydrogen bonding with the solvent resulting in three sharp peaks observed in low-field signal. These three sharp NH signals were the most striking features of violacein as reported by Hoshino et al. (1987) and Yada et al. (2007). The numbering of the structure was cited from Nakamura et al. (2002) (Fig. 2.9).

The ^{13}C NMR spectrum exhibited the presence of 20 carbon atoms (Fig. 2.10). The carbonyl carbons were detected at δ 170.7 ppm (C-16) and δ 172.1 ppm (C-11) while 18 other carbons were observed at δ 97.5 (C-13), 105.1 (C-5), 106.2 (C-3), 109.5 (C-22), 113.6 (C-7), 113.9 (C-8), 119.2 (C-17), 121.3 (C-20), 122.9 (C-18), 126.1 (C-4), 126.8 (C-19), 129.9 (C-2), 130.1 (C-21), 132.1 (C-9), 137.5 (C-12), 142.3 (C-23), 148.1 (C-14) and 153.4 (C-6). The signal at δ 153.4 indicates

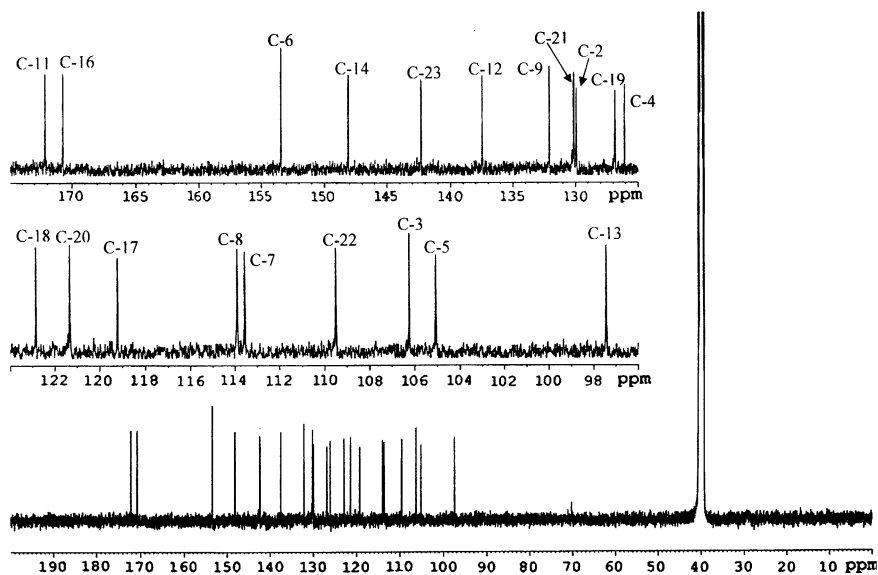


Fig. 2.10 ^{13}C NMR spectrum of violet pigment

that the pigment was violacein, since the signal for corresponding carbon of deoxyviolacein, which lacks hydroxyl residue, appears in the higher magnetic field (Hoshino et al. 1987).

Powdered form of the crude extract (0.5 g) was dissolved in DMSO prior to analysis using the Electron Spray Ionization–Mass Spectrometry (ESI–MS) which was carried out at the National Metrology Laboratory, Standard and Industrial Research Institute of Malaysia (SIRIM), Salak Tinggi, Malaysia. A quasimolecular ion peak was observed at m/z 342.34 $[\text{M}-\text{H}]^-$ (Fig. 2.11) while the MS/MS spectrum (Fig. 2.12) showed the ion fragments at m/z 298.42, m/z 209.07 and m/z 157.16. These clearly correspond to the molecular formula of $\text{C}_{20}\text{H}_{13}\text{O}_3\text{N}_3$, which together with the NMR and FTIR analysis, strongly suggests that the violet methanolic pigment extract is violacein. Similar conclusion was made for violet pigment isolated from other bacteria such as *Janthinobacterium lividum* (Lu et al. 2009), *Duganella* sp. B2 (Wang et al. 2009), *Pseudoalteromonas luteoviolacea* (Yada et al. 2007) and *Alteromonas luteoviolacea*.

Antibacterial activities

The agar disc diffusion method was used to determine the antibacterial activity of the crude-violet pigment as follows (Barja et al. 1989); *Bacillus cereus* (*B. cereus*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) were incubated in NB medium either at 30 or 37 °C for 12 h at 200 rpm. Then, 0.1 mL of the culture broth was inoculated onto NA plates containing Whatman filter paper discs (wet strengthened, 0.5 cm in diameter) which were previously impregnated with 50 μL of the crude methanolic extract. Inhibition zones were recorded after overnight incubation at either 30 or 37 °C, respectively.

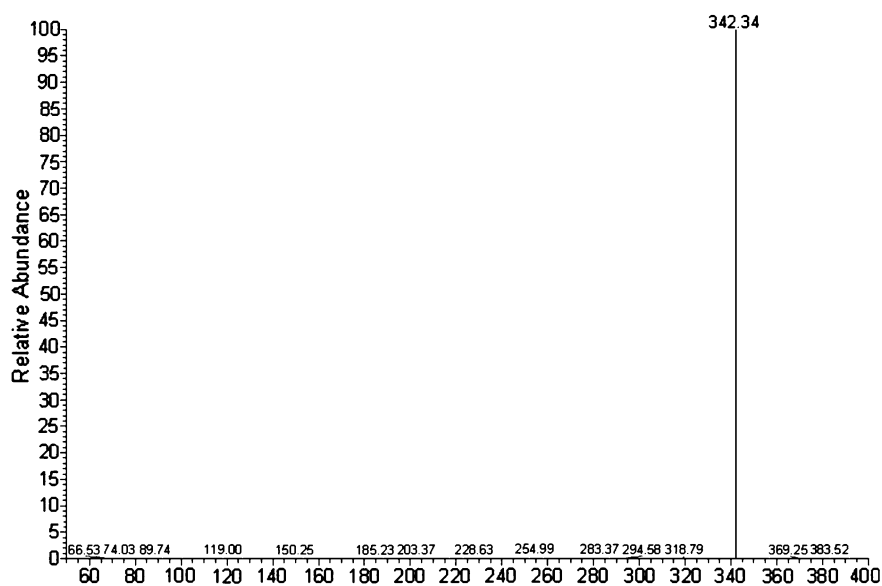


Fig. 2.11 The ESI-MS spectrum of violet pigment

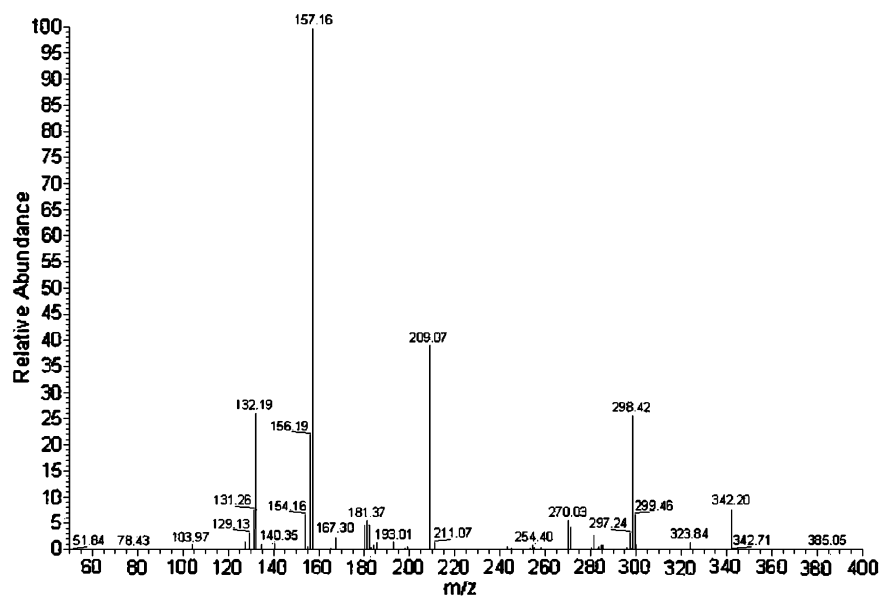
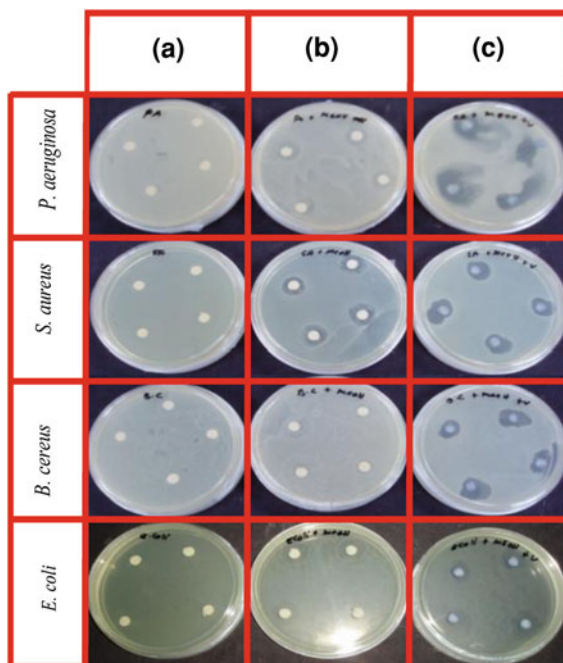


Fig. 2.12 The ESI-MS/MS spectrum of violet pigment ($m/z = 342.34$)

Fig.2.13 Antimicrobial testing of violet pigments on *P. aeruginosa*, *S. aureus*, *B. cereus* and *E. coli*; (a) – Control (bacteria only); (b) – bacteria + methanol (medium suspension for pigment); (c) – bacteria + methanol + violet pigment



Similar experimental setup consisting of filter paper discs impregnated with 50 μ L methanol and filter paper discs only acted as control. The appearance of the inhibition zone on both the Gram-positive (*S. aureus* and *B. cereus*) and the Gram-negative bacteria (*P. aeruginosa* and *E. coli*) indicated that the violet pigment has a broad-spectrum antibacterial property (August et al. 2000) (Fig. 2.13). A slightly larger inhibition zones for the Gram-positive bacteria suggested different anti-bacterial efficiency level of the violet pigment depending on classes of bacteria tested. Among the most important factors influencing the efficacy of antibacterial agents include nature of physical environment and condition of microorganism (Bloomfield 1991), rigidity of the bacterial structure as well as inhibition of the necessary metabolic reaction in a microorganism (Nakamura et al. 2003).

2.6.3 Characterization of Yellow–Orange Pigment

An overnight grown culture of *Chryseobacterium* sp. was centrifuged and the pellet was deposited on a glass slide placed on a white background, then flooded with potassium hydroxide (KOH), 20 % (w/v) and the color shift was observed. The resulting color may then be compared with the initial color of the pellet which acted as a control. After removing excess KOH, an acidic solution was flooded to revert their initial color (Bernardet et al. 2002). The *Flavobacteriaceae* group

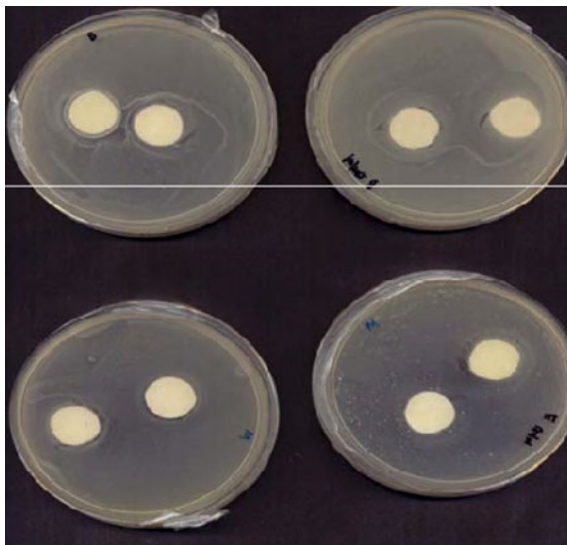
was reported to produce yellow to orange (or rarely red) pigmentation. As *Chryseobacterium* sp. falls into this group and it produces yellow–orange pigment, a simple test for flexirubin-type pigment was done to diagnose the type of pigment. In this test, a plate containing the *Chryseobacterium* sp. was scrapped and flooded with 20% (w/v) KOH. From the observation, the color of the colony changes from yellow–orange to red–brown. The resulting color was then compared with the bacteria which were not in contact with KOH. After removing the excess KOH, an acidic solution was flooded to revert their initial color. It was suggested that flexirubin reaction was shown in the presence of acid and base condition because it is one diagnostic feature of flexirubin type pigment (Balows et al. 1992). Similar finding was obtained by Kim et al. 2005, where the Flexirubin-type pigment was detected in *Chryseobacterium daecheongense* sp. nov. according to this method. According to Bernardet et al. 2002, since the color shift may pass unnoticed when the KOH solution is poured directly over a thin colony on an agar plate, it is strongly recommended that the test be performed on a small mass of bacterial cells collected with a loop and deposited on a glass slide placed on a white background. Another similar mass of bacteria on which no KOH is poured may be used as a control. The color changed induced by KOH is not absolutely specific for the flexirubin-type of pigment (Fautz and Reichenbach 1980), but it is still helpful when combined with the results of other tests. Natural yellow pigment was tested against common pathogens *Escherichia coli* which is a Gram-negative bacteria and *Bacillus cereus* which is a Gram-positive bacteria. By using the qualitative method, the yellow pigment inhibited the growth of *Bacillus cereus* and *Escherichia coli*. The antibacterial activity of a material depends on the destruction of the physical structure or the inhibition of reaction in a microorganism; it seems that the presence and the level of the antibacterial activity of the pigment varied significantly with the type of microorganism used (Martinko and Madigan 2006). It can be seen from Fig. 2.14 that the yellow pigment has some antimicrobial activity as evidenced from the clearing zones observed in the plates containing *Bacillus cereus* and *Escherichia coli*. To have an idea of the degree of antimicrobial activity, the spread plate method was carried out. A greater percentage kill was observed for the Gram-positive bacteria as compared to the Gram negative bacteria (15 % and 55.61 %, respectively). This could be due to the thinner peptidoglycan layer found in the Gram-negative bacteria making it more susceptible to the pigments.

2.6.4 Characterization of Red Pigment

Extraction and purification

A modified procedure for the isolation of prodigiosin, a known red pigment produced by *S. marcescens*, was carried out according to Wei et al. (2005); a 24 h old culture broth (1.5 liters), incubated at 25 °C and 200 rpm, was mixed with 4 liters of 95 % (v/v) methanol and mixed vigorously via vortex mixing.

Fig. 2.14 Anti-microbial testing of yellow pigments on *B. cereus* and *E. coli*



The mixture was then centrifuged at 6854 rpm, 0 °C for 10 min. The resulting supernatant was collected and filtered through a 0.2 μm Whatman filter paper. The filtrate was concentrated using a rotary evaporator (Rotavapor R-200, Buchi) followed by extraction using 5.0 mL chloroform. The chloroform extract was reconcentrated using rotary evaporator until minimal volume was obtained. This minimal volume of chloroform extract was then transferred into a glass petri dish prior to drying in a vacuum drying oven. Purification of the crude product was carried out using column chromatography on a silica gel (230–450 μm , Sigma) column (30 cm height, 2.5 cm internal diameter, 2.9 cm outer diameter). The column content was first washed with hexane. The dried crude product was dissolved in 15 mL of 95% (v/v) methanol before passing through the column. The crude extract - loaded column was eluted with 10.2 M ethyl acetate. Every 8 mL of the orange eluate fractions were collected and examined for the presence of prodigiosin by thin layer chromatography, TLC (silica gel 60 F₂₅₄, Merck) using ethyl acetate as solvent. Fractions having a single spot were pooled and evaporated in a desiccator to obtain the purified product.

UV-vis, FTIR and NMR analysis

The pigment was analyzed for maximum UV-vis absorbance at pH values of 2, 7 and 9 between 800 and 200 nm, using methanol as blank. FTIR spectroscopy was carried out by mixing the vacuum-dried pigment with finely ground KBr (1:100). Upon pressing under 2,000 kPa, pellet disc obtained was analyzed using a FTIR spectrophotometer (FTIR 8300, Shimadzu) between 4,000 and 400 cm^{-1} . The pigment (5–10 mg) was dissolved in 500 μL of chloroform-*d*₂ (CDCl₃) for NMR analysis (Min-jung et al. 2006). UV-vis analysis spectra for the red pigment in methanol showed a maximum peak at 534.76 nm which closely resembles the reported maximum absorbance for prodigiosin, i.e., 535 nm (Song et al. 2000).

Fig. 2.15 Different coloration of the red pigment at (a) pH 2 (b) pH 7 and (c) pH 9

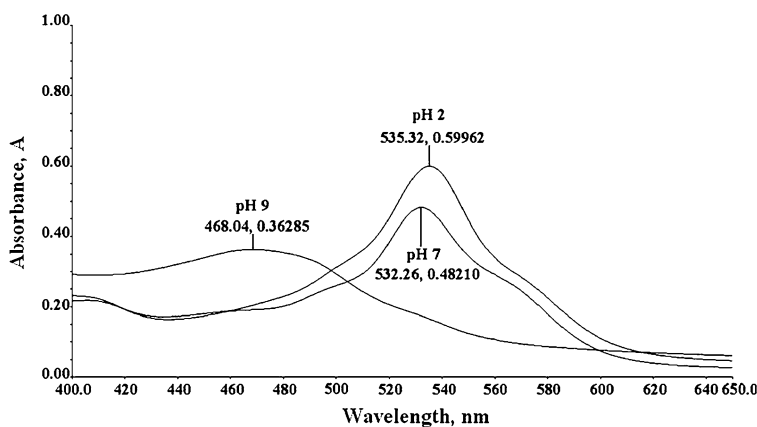
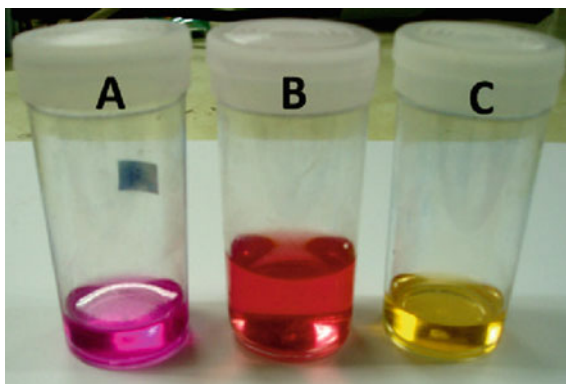


Fig. 2.16 Maximum absorption of the red pigment at pH 2, 7 and 9; pigment dissolved in methanol

The red-pigment changes to pink at pH 2, orange at pH 9 while retaining its red feature at pH 7 (Fig. 2.15). Similar observation was previously reported by Min-jung et al. (2006). At pH 2, maximum absorbance for the pigment was recorded at $\lambda_{\max}$ 535.32 nm, 532.26 nm at pH 7 and 468.04 nm at pH 9 (Fig. 2.16).

The FTIR spectra of the red pigment showed that it has several degree of similarity to the spectra of prodigiosin (Song et al. 2000; Rustom et al. 1990). The summary of the important functional groups found in prodigiosin is as shown in Table 2.4.

The similar peaks obtained for the red pigment are as shown in Table 2.5. The main peaks are at 3445.45, 2926.23, 1718.04, 1650.96, 1559.85, 1537.95, 1508.44, 1458.75 and 1072.49 cm^{-1} . From the peaks, it was suggested that the main functional group for the red pigments are pyrrole, methylene, alkane and alkene.

The significant ^1H NMR (CDCl_3) chemical shifts obtained for the red pigment are as follows; 0.0826 mg L^{-1} , 0.5846 mg L^{-1} , 0.6522 mg L^{-1} , 0.8734 mg L^{-1} , 1.2637 mg L^{-1} , 1.3756 mg L^{-1} , 1.6071 mg L^{-1} , 2.0478 mg L^{-1} , 2.3585 mg L^{-1} ,

Table 2.4 Band assignments to the functional groups present in prodigiosin

Wave number (cm ⁻¹)	Band assignment	Reference
2852	Methylene	Song et al. (2000)
1714	–	
1630	–	
1604	–	
1545	Pyrrole ring structure	Rustom et al. (1990)
3500	Amide groups	
2800	Methylene	
1500, 1565	Pyrrole ring structure	

Table 2.5 FTIR band assignments for red pigment

Wave number (cm ⁻¹)	Band assignments	Wave number (cm ⁻¹)	Band assignments
3445.45	Aliphatic alcohols (s) Primary amines (m), amide (m)	1537.95	C=C stretching vibrations
2926.23	C–H stretching (s) Methylene	1508.44	Secondary amines (w) Azo compounds (w) Aromatic (m)
1718.04	C=O stretching vibrations Ester (s) Aryl aldehydes (s) Saturated ketone (s) Saturated carboxylic acid (s)	1458.75	C–H bending vibrations (m) Ethylenediamine complex (m) Azo compounds (w)
1650.96	C=C Stretching Vibrations Aliphatic amines (m) Enol (s)	1072.49	C–H out-of-plane bending vibrations Aliphatic amine (w-m)
1559.85	Secondary amines (w)		

m moderately strong, *s* strong, *w-m* weak to moderately strong, *w* weak

3.6781 mg L⁻¹, 3.8127 mg L⁻¹, 4.1521 mg L⁻¹ and 5.3590 mg L⁻¹. Shift for the solvent was at 7.2734 mg L⁻¹. The main functional groups observed were: saturated alkane, amine, methyl (primary), methyl (secondary), methyl (tertiary), esters, acetylenic, ethers, alcohols and vinylic. It was observed that the signals of the vinylic functional group (C=C) were very weak in the NMR spectra.

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