

Original Research Article

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Pyomelanin Production From a Marine Isolate of *Acinetobacter Spp.*

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ABSTRACT

There is growing interest in the use of natural pigments for a wide range of applications. A search for pigment-producing bacteria led to the isolation of a marine bacterium that produced a diffusible brown pigment. The bacterium was identified as *Acinetobacter spp.* from 16S rRNA gene sequencing. The pigment displayed free-radical scavenging activity and was characterized as a melanin through solubility tests, UV-Vis spectrophotometry and Fourier transform infrared (FTIR) spectroscopy. Melanin production was blocked by sulcotrione, an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD), the enzyme responsible for producing homogentisic acid (HGA), which is the monomeric precursor of pyomelanin. Inactivation of melanin synthesis by HPPD inhibition confirmed that the pigment was pyomelanin. *Acinetobacter spp.* required at least 12 days to produce approximately 1g/L of pyomelanin when it was grown in basal medium supplemented with L-tyrosine. The rate of pyomelanin production was not influenced by nutrient levels in growth media. Supplementation with different carbon sources and the use of nutrient-rich media had no effect in accelerating pigment production. However, pH had a major effect on pyomelanin formation by significantly reducing the time taken to produce the pigment. When the pH of growth medium (pH 5.5) was adjusted to pH 8, bacteria were able to produce pyomelanin in 7 days instead of 12-14 days.

Keywords

Bacterial pigments,
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Introduction

The global shift away from manufacturing synthetic and convenient products to natural and sustainable products has been accelerating over recent years. An emerging area is the use of biological pigments as alternatives to synthetic ones (Venil *et al.*, 2013). Pigments are essential colorants in many industries including food and beverage, paint, plastics, cosmetics, clothing, printing

and many others. The list of reasons for discontinuing the use of synthetic pigments is growing and includes its potential toxicity to plant, animal and human populations, widespread environmental contamination and negative impact on ecosystems (Chung, 2016; Lellis *et al.*, 2019; Tkaczyk *et al.*, 2020).

Natural pigments are less harmful alternatives and they possess additional useful characteristics such as antioxidant, anti-UV

(ultraviolet), antimicrobial, anti-fungal and anti-tumour activities that have not been fully exploited (Venil *et al.*, 2013). Natural pigments are widespread and can be found in plants, animals, fungi and bacteria but bacterial pigments may be the most attractive in terms of production on a commercial scale. Although plant pigments are the most widely used of the biological pigments with a wide range of applications (Mansour, 2018), producing pigments from bacteria has several advantages. The rapid life cycle of bacteria allows for easy and frequent pigment extractions, the cultivation of bacteria can be readily scaled up in a controlled environment that is free from seasonal and climatic variations, pests and disease. Moreover, bacteria are readily amendable for strain improvement via genetic manipulation (Malik *et al.*, 2012). Hence, there has been growing interest in the application of bacterial pigments as therapeutic and medicinal agents, non-toxic inks for printing, dyes for food and textiles and colorants for cosmetics (Venil *et al.*, 2013).

We report here the isolation, culture and identification of a marine isolate of *Acinetobacter spp.* recovered from seawater off Singapore. The bacterium produced a diffusible dark-brown pigment, subsequently identified as pyomelanin. Pyomelanin is one of several types of melanins, which are heterogeneous polymers of phenolic or indolic compounds produced by a wide variety of prokaryotes and eukaryotes (Nosanchuk and Casadevall, 2003; Plonka and Grabacka, 2006). Melanins have several properties in common, including the ability to absorb ultraviolet (UV) light, ability to scavenge free radicals, inability to dissolve in aqueous or organic fluids, resistance to concentrated acid and susceptibility to bleaching by oxidizing agents (Bustamante *et al.*, 1993; Nosanchuk and Casadevall, 2003). Pyomelanin is a dark-brown pigment formed from the accumulation

of homogentisic acid (HGA), an intermediate molecule in the degradation of phenylalanine and tyrosine. HGA can autooxidise and self-polymerise into pyomelanin, a molecular structure of unknown organization and size (Turick *et al.*, 2010). This study provides a description of the production, characterization and subsequent identification of pyomelanin from an isolate of *Acinetobacter spp.* and the effect of pH on pyomelanin production.

Materials and Methods

Isolation and culture of bacteria

Pigment-producing bacteria were isolated from seawater off the North-East coast of Singapore with basal agar medium containing 0.5% (w/v) L-tyrosine. Agar plates were incubated at 30°C aerobically for 48 h and examined for pigment-producing colonies. A pigment-producing colony, named PRSW10118-BK (BK), was selected for further study. Thereafter, bacteria were cultivated on either nutrient agar/broth or basal medium with 0.5% L-tyrosine and grown at 35°C aerobically. Basal media containing 0.5% L-tyrosine was prepared according to Yabuuchi and Ohyama (1972). Agar Bacteriological (1.5% w/v) (Oxoid, UK) was added to the liquid medium to make basal agar medium. Nutrient broth (Oxoid, UK) was prepared according to the manufacturer's instructions, with 0.5% L-tyrosine. All media were sterilized by autoclaving at 121°C for 20 min before use.

DNA extraction and 16S rRNA gene sequencing

Bacterial DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) as described in the manufacturer's instructions. The 16S rRNA gene was amplified and sequenced using bacterial universal primers 27F (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in 25 μ L volumes consisting of genomic DNA (50 ng), 1 μ L forward primer (12.5 μ M), 1 μ L reverse primer (12.5 μ M), 12.5 μ L 2 \times Phusion High-Fidelity PCR Master Mix (Thermo Scientific) and adjusted to 25 μ L with nuclease-free water. PCR was performed in an ABI/Perkin Elmer 9600 PCR machine with the following cycling conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 2 min.

A final extension was performed at 72°C for 10 min. PCR products were visualized in a 1% agarose gel, stained with Sybr Green and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Sanger sequencing was performed by Bio Basic Asia Pacific Pte Ltd (Singapore). DNA sequences were then analyzed using BLAST available from the National Center of Biotechnology Information (NCBI) website.

Production and extraction of bacterial melanin

To facilitate melanin recovery and minimize contamination of the final melanin product, basal and not nutrient-rich media, supplemented with 0.5% L-tyrosine, was used to grow the bacteria. Inoculated flasks were placed in a shaking incubator (150 rpm) at 35°C until the medium turned dark-brown (at least 12 days). Cultures were then centrifuged at 7000 \times g (Beckman, JAL-16500) for 30 min. The supernatant was recovered and the pH was adjusted to pH 2 with 6M HCl and left to stand for at least 2h. The precipitate was then thoroughly washed three times with generous volumes (220 mL) of sterile MilliQ water. Centrifugation was carried out at 7000 \times g for 10min each wash. The purified pigment was then dried in a 60°C oven

overnight and weighed. To assess whether bacterial growth and pigment synthesis could be accelerated, the basal medium was supplemented with different carbon sources. Basal medium containing 0.5% L-tyrosine was supplemented with 1% of the following: glucose, sucrose, mannose, glycerol, D-sorbitol, fructose or maltose in separate flasks. The pH of basal medium was adjusted from pH 5.5 to pH 6, 7 or 8 to assess whether pH had any effect on the time required to synthesize the pigment.

Solubility and oxidizability tests

The ability of the pigment to dissolve in a variety of solvents and be bleached by hydrogen peroxide was assessed using the methods described by Guo *et al.*, (2014). Solvents included 100% methanol, 100% ethanol, 99% acetone, phenol, chloroform, sodium hydroxide (1M) and dimethyl sulfoxide (DMSO). To determine whether the pigment could be oxidized by hydrogen peroxide, a 0.01% melanin solution was mixed with H₂O₂ at different final concentrations (1, 2 and 5%). Absorbance was measured using a Shimadzu, UV-1800 spectrophotometer.

UV-Vis spectroscopy

A 0.005% (w/v) solution of bacterial melanin was made by dissolving extracted pigment in 0.1M NaOH. The melanin solution was subjected to a spectral scan using a Shimadzu, UV-1800 spectrophotometer at 200 – 800 nm to determine peak absorbance. Synthetic melanin (Merck, USA) was prepared and analysed in the same manner.

FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to analyse the bacterial melanin against eumelanin from cuttlefish

(*Sepia officinalis*). Melanin was ground with infrared quality dry KBr and pressed into a pellet with an MHP-1 Pelletizer. The infrared (IR) spectrum (4000-400 cm^{-1}) was recorded with a Shimadzu IR Prestige-21 NIR system and AIM 8800 IR microscope.

Free-radical scavenging assay

A 0.004% (w/v) DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by dissolving DPPH powder in absolute methanol. The test samples, at concentrations of 20, 40, 60, 80, 100, 120, 140 $\mu\text{g/mL}$ were prepared by dissolving dried pigment in 10mM Tris-HCl (pH 8). One hundred microliters of sample was mixed with 100 μL of DPPH solution in a 96-well flat bottom microtitre plate (Greiner Bio-One, Germany). Absorbance was measured at 520nm after the mixture was left at room temperature in the dark for 30 min. Ascorbic acid was used as the positive control. The strength of scavenging activity was expressed as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Specific inhibitors of melanin synthesis

Sulcotrione and kojic acid, known inhibitors of 4-Hydroxyphenylpyruvate dioxygenase (HPPD) and tyrosinase/laccase, respectively, were used to determine the identity of the bacterial melanin. Sulcotrione was added to tyrosine-supplemented basal agar medium at final concentrations of 10, 20 and 50 μM .

Kojic acid was added to tyrosine-supplemented basal agar plates at final concentrations of 50 μM , 100 μM and 200 μM . Plates were streaked with the bacterial isolate BK and incubated aerobically at 35°C and visually inspected after 48 h.

Results and Discussion

Identification of the pigment-producing bacterium

The pigment-producing bacterium isolated from seawater off Singapore was identified as *Acinetobacter spp.* through 16S rRNA gene sequencing. Electropherograms of the forward and reverse sequences were checked and verified using Chromas 2.6.6. Both forward and reverse sequences were aligned with sequences in GenBank by the BLAST algorithm, which matched the sequences to *Acinetobacter venetianus* (95% identity). We have therefore classified bacterial isolate BK as *Acinetobacter spp.*

Characterization and identification of the bacterial pigment

Acinetobacter spp. isolate BK produced an extracellular diffusible pigment, which could be precipitated and extracted from an acidified solution (Fig. 1). After 12-14 days of growth, on average, the yield of bacterial pigment was approximately 1 g/L in basal medium containing L-tyrosine. The extracellular pigment was identified as a melanin from its solubility profile, UV-Vis absorption spectrum and FTIR spectroscopy. The pigment was insoluble in water and a number of solvents but could be dissolved in alkali solution. It was soluble in dimethylsulfoxide (DMSO) and partially soluble in methanol (Table 1). Moreover, it could be decolourized with H_2O_2 , which is consistent with other bacterial melanins described previously (Chen *et al.*, 2004; Sajjan *et al.*, 2010; Guo *et al.*, 2014). Bacterial melanin from *Acinetobacter spp.* BK strongly absorbed light in the UV region, peaking at 215 nm. Synthetic melanin produced an identical peak in the same region (Fig. 2). The spectral scan of the pigment from *Acinetobacter spp.* BK is typical of most

melanins, showing a strong optical absorbance in the UV region that decreased towards longer wavelengths. Peak absorption of most melanins ranges between 196–300 nm, depending on the melanin source (Pralea *et al.*, 2019). Melanin is well known for possessing antioxidant activity (Bustamante *et al.*, 1993; Jacobson *et al.*, 1995). To assess whether the pigment displayed antioxidant activity, it was subjected to a free-radical scavenging assay using the stable free radical DPPH. Absorbance of the purple solution of DPPH decreased as it turned pale yellow as a result of losing electrons scavenged by the pigment. As the concentration of melanin increased, its free-radical scavenging activity also increased and then plateaued at concentrations $\geq 100 \mu\text{g/mL}$, as did ascorbic acid (Fig. 3). Although not as strong an antioxidant as ascorbic acid, the pigment from *Acinetobacter spp.* BK clearly displayed free radical scavenging activity.

FTIR spectroscopy was used to determine the functional groups and fingerprint regions in the melanin (Fig. 4). Both the bacterial melanin and eumelanin displayed peaks that were reflective of structures expected to be found in melanin. Firstly, both pigments displayed similar peaks in the functional group region with broad peaks at 3294.42 and 3387.00 cm^{-1} , respectively, which is characteristic of O-H stretching (Coates, 2000). For the bacterial melanin, the peaks at the following wave numbers and their corresponding structures were: 2931.80 aliphatic C-H stretching; 1651.07 aromatic C=C conjugated with C=O and/or COO⁻ groups; 1535.34 aromatic C=C bonds; 1450.47 aliphatic C-H; 1226.73 phenol C-O stretching; 1056.99 primary alcohol C-O stretching (Coates, 2000; Turick *et al.*, 2002; Singh *et al.*, 2018). For cuttlefish eumelanin, the peak at 1597.00 could be attributed to carbonyl stretching in indole quinone; 1355.60 cm^{-1} could be from bending O-H and

NH bonds combined with various modes of aromatic rings (Glass *et al.*, 2012). Although production of eumelanin has been described in bacteria (Plonka and Grabacka, 2006), the clear difference between *Acinetobacter* melanin and eumelanin in the fingerprint region, at and below 1651.07 cm^{-1} , strongly suggested that the *Acinetobacter* melanin could not be eumelanin.

This was further supported by results from inhibition tests with kojic acid, a chelator of copper ions and inhibitor of copper-containing enzymes like tyrosinase and laccase, which failed to block melanin production by *Acinetobacter spp.* isolate BK. Tyrosinase is an enzyme involved in the biosynthesis of eumelanin (Plonka and Grabacka, 2006). However, when sulcotrione, a specific inhibitor of HPPD, was added to growth media, it was able to completely inhibit melanin synthesis (Fig. 5). HPPD is the enzyme responsible for converting 4-hydroxyphenylpyruvic acid into HGA, the monomeric precursor of pyomelanin (Turick *et al.*, 2010). This was conclusive evidence that the pigment produced by *Acinetobacter spp.* BK was indeed pyomelanin.

Pyomelanin is formed from HGA, which is produced from the degradation of phenylalanine and tyrosine. Tyrosine is converted to 4-hydroxyphenylpyruvic acid then HGA, which is excreted from bacterial cells where it autoxidizes into benzoquinoneacetic acid and self-polymerises into pyomelanin (Turick *et al.*, 2010). *Acinetobacter spp.* BK required 12-14 days to turn the basal medium, with an initial pH of 5.5, completely dark brown. The pH of the growth medium at day 12 was found to range from pH 8.1 to 8.7. The addition of glucose, sucrose, mannose, glycerol, D-sorbitol, fructose and maltose as the sole carbon source to the basal medium, did not accelerate the formation of pyomelanin (data not shown).

Table.1 Solubility and oxidizability of melanin from *Acinetobacter spp.* BK

Test	Treatment	Result
Solubility	H ₂ O	Insoluble
	HCl (1M)	Insoluble
	Acetone	Insoluble
	Chloroform	Insoluble
	Ethanol	Insoluble
	Phenol	Insoluble
	Methanol	Partially soluble
	Dimethylsulfoxide	Soluble
	NaOH (1M)	Soluble
Oxidizability	1%, 2%, 5% H ₂ O ₂	Decolorized (dark to near colourless)

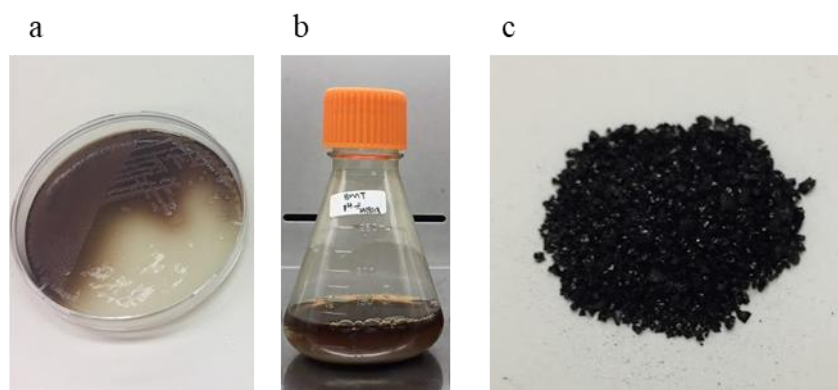
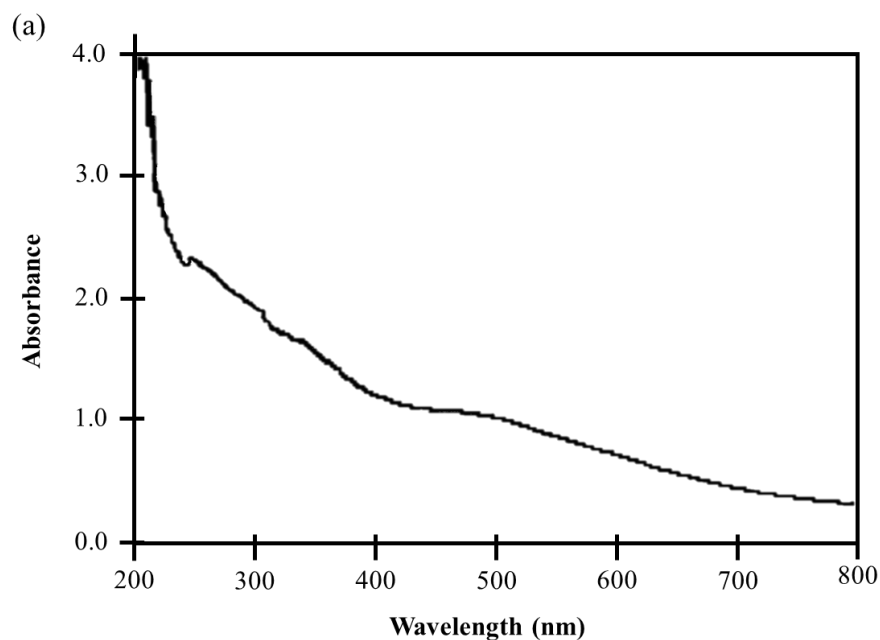


Fig.1 *Acinetobacter spp.* BK producing diffusible brown melanin on (a) agar and in (b) basal medium containing 0.5% (w/v) L-tyrosine. (c) The melanin was extracted by precipitation in acid and washed thoroughly with water



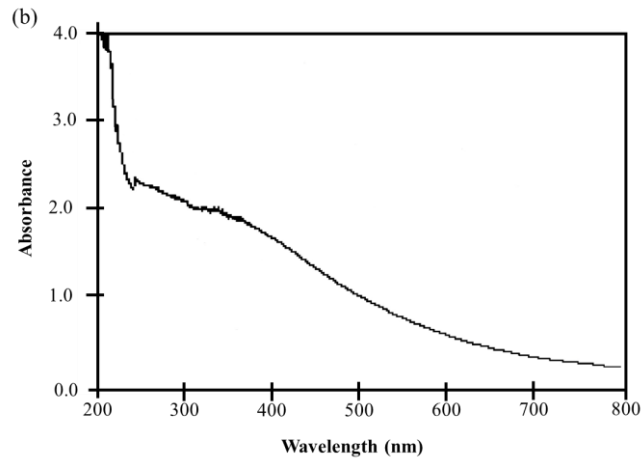


Fig.2 Absorption spectra of (a) melanin produced by bacterial isolate BK and (b) synthetic melanin. Peak absorption was in the UV region at 215 nm for both pigments

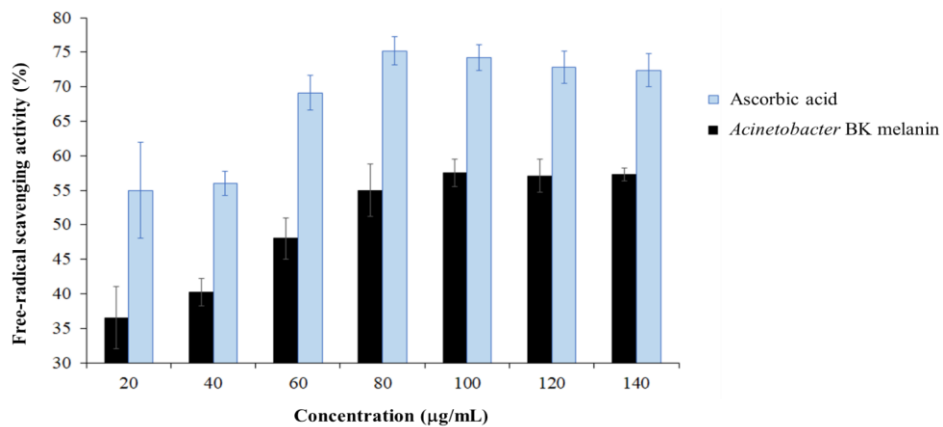


Fig.3 DPPH scavenging activity of melanin from *Acinetobacter spp.* isolate BK compared to ascorbic acid, a known antioxidant

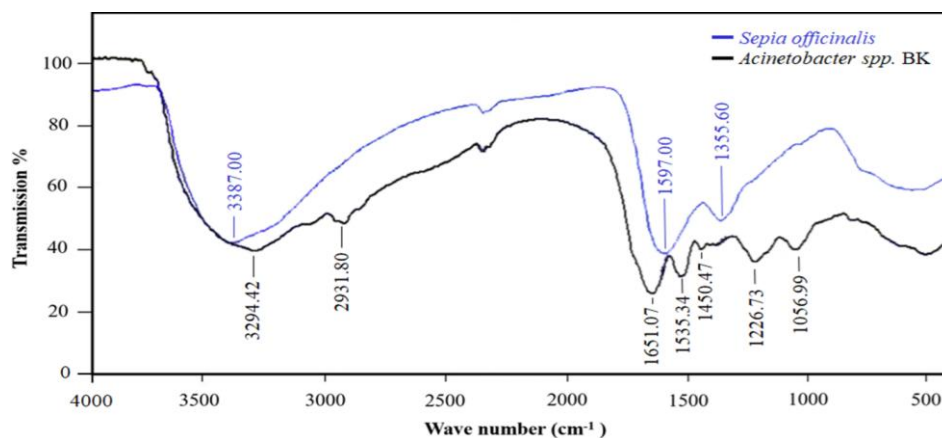


Fig.4 Fourier transform infrared scans of melanin isolated from *Acinetobacter spp.* isolate BK and eumelanin from *Sepia officinalis* (cuttle fish). The pigments showed some similarity in the functional group region but differed significantly in the fingerprint region, encompassing peaks with wave numbers $\leq 1651.07 \text{ cm}^{-1}$

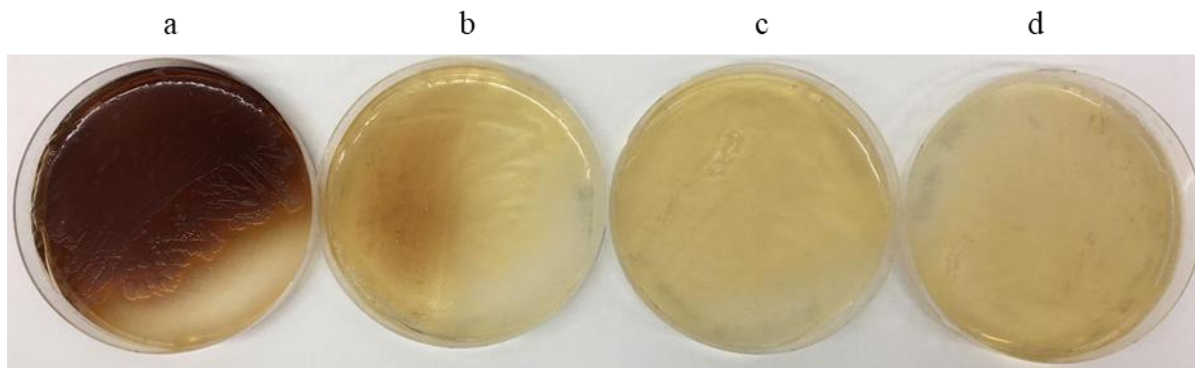


Fig.5 *Acinetobacter spp.* BK grown on basal agar supplemented with 0.5% L-tyrosine, containing sulcotrione at (a) 0 mM (b) 10 mM (c) 20 mM and (d) 50 mM. Melanin synthesis was partially inhibited at 10 mM but was completely inhibited at concentrations ≥ 20 mM

Moreover, nutrient-rich broth (pH 7) containing L-tyrosine, which should have accelerated growth of the bacterium also took at least 12 days for the broth to turn dark brown. The level of nutrients in the media had no effect on the time taken to produce pyomelanin. However, pigment formation could be accelerated when the starting pH of the basal medium was increased from pH 5.5 to 8, which reduced the production time from 12-14 to 7 days. There was no difference between media at pH 5.5 and pH 6 and only a slight reduction to 11-12 days at pH 7 (data not shown). Lagunas-Muñoz *et al.*, (2006) described a similar positive effect of alkaline pH on eumelanin synthesis in recombinant *E. coli* cells, where a slightly alkaline pH promoted autooxidation and polymerization of eumelanin. They found that at pH 7 there was a drastic decrease in eumelanin production after 12–18 h of the initial production stage but the rate of eumelanin formation was maximal at pH 7.5 – 8 (Lagunas-Muñoz *et al.*, 2006).

Visible pyomelanin took longer to form in liquid basal medium (pH 5.5; at least 12 days) compared to solid agar medium (2 days). The initial acidic pH of the liquid basal medium probably contributed to the extended time required for pyomelanin production because acidic conditions are unfavourable to melanogenesis and may even inhibit melanin

synthesis if the pH ≤ 5.5 (Ancans *et al.*, 2001). It may have taken well over a week for *Acinetobacter spp.* to secrete enough metabolites into the growth medium to raise the pH to a level that is conducive for melanin formation. Another limiting factor may be the amount of HGA present in the medium for melanin polymerization to occur. In broth, due to the larger volume compared to agar plates, it takes much longer to accumulate enough monomeric HGA to polymerize into pyomelanin. HGA is only excreted out of the bacterial cell when its production exceeds that of HGA-oxidase activity (Turick *et al.*, 2010). If HGA production does not exceed HGA-oxidase activity then HGA is converted to 4-maleylacetoacetic acid and no pyomelanin is formed. Therefore, it may require many days for significant amounts of HGA to accumulate in the medium such that it is sufficient for assembly of macromolecular pyomelanin.

Bacteria are increasingly used as biofactories to produce biological products. Natural pigments like pyomelanin could easily be produced and extracted from bacteria. However, to optimize pigment production it is important to understand the environmental conditions that favour pigment formation as well as the biochemical pathway leading to its synthesis.

The present study demonstrated the effect of alkaline pH in reducing the time it takes to produce pyomelanin. To successfully produce the pigment on commercial scale it may be necessary to also consider overcoming the limiting amounts of excreted HGA. This could be done by employing genetic mutants with a modified biochemical pathway that is directed at overproducing HGA.

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