Isolation and characterization of flexirubin type pigment from *Chryseobacterium* sp. UTM-3T

Chidambaram Kulandaisamy Venila, Zainul Akmar Zakaria, Rajamanickam Usach, Wan Azlina Ahmad

1. Introduction

The present trend throughout the world is shifting towards the use of eco-friendly and biodegradable natural colorants due to its pharmacological activities. The demand for natural colorants is increasing day by day and are sourced from ores, insects, plants and microbes. Among microbes, bacteria have the immense potential to produce diverse bioproducts and one such bioproduct is pigment. The production and application of bacterial pigment as natural colorants is being investigated by various researchers (Venil et al., 2013). Pigments produced by bacteria are of traditional use in oriental countries and have been a subject of intense research in the present decades because of its potential for applications. Most researchers have focused on the production of yellow, violet and red pigment production from different bacteria. However, the study of pigment (flexirubin) from *Chryseobacterium* is very limited.

Flexirubins are the unique type of bacterial pigments with terminal alkyl substitution consisting of \( \omega \) -phenyl octaenic acid chromophore esterified with resorcinol and are used in the treatment for chronic skin disease, eczema, gastric ulcers etc (Kim, 2013). Recent studies of the genus *Chryseobacterium* have documented the significance of its bioactive compounds as bio-control agent, antioxidants, prebiotics, sulfobacin A and protease producer (Scheuplein et al., 2007; Wang et al., 2007; Chaudhari et al., 2009; Wang et al., 2011; Kim et al., 2012) which substantiate that it is a novel source of bioactive compounds. The flexirubin was first isolated in 1974 from *Flexibacter elegans* (Reichenbach and Kleining, 1974) which turned out to be member of a novel class of pigments (Achenbach et al., 1974, 1976). Similar pigments were later identified in a number of other bacteria viz., *Cytophaga* sp, *Sporocytophaga* sp and *Chryseobacterium* sp. Each genera produce specifically modified species of flexirubin which serve as excellent chemosystematic markers (Reichenbach and Kleining, 1974). According to the survey on the occurrence of the novel compounds, the distribution of flexirubin is rather limited. In the present study, the pigment from *Chryseobacterium* sp. UTM-3T was characterised and the properties of the pigment were studied to reveal its potential as a substitute for synthetic colorants.

2. Materials and methods

2.1. Bacteria, media and culture conditions

The pigmented bacteria, *Chryseobacterium* sp. UTM-3T (=CECT 8497T=KCTC 32509T) isolated from the Orchard in Universiti Teknologi Malaysia, Malaysia was used in this study. The 16S rRNA
The dried bacterial pellet (1 mg) was analyzed using the Perkin Elmer Precisely Lambda 25 Vis spectrophotometer (Perkin Elmer Precisely Lambda 25) between 800 and 200 nm. All chemicals used were of analytical grade. The bacterial cell was cultivated in 20 mL NB in 100 mL Erlenmeyer flask at 30 °C and 200 rpm agitation speed until it reached 1.0 optical density at 600 nm (OD600). 2 mL of the culture was transferred as inoculum into 100 mL of the fresh medium in 500 mL Erlenmeyer flask and incubated at 30 °C at the shaking speed of 200 rpm for 24 h and the pigment yield was calculated.

2.2. Isolation and purification of the pigment

The culture broth was centrifuged at 8000 rpm for 10 min and the supernatant was discarded. The cell pellet was then rinsed with deionized water, followed by centrifugation at 8000 rpm for 5 min to recover the cells by decanting the supernatant. The recovered cells were extracted using 5% acetone according to the method of Williams et al. (1956). The mixture of cells and acetone was treated by ultrasonication until the cells were completely bleached. The pigment was then separated from the cells by centrifugation at 10,000 rpm for 5 min. By this method, it was confirmed that there was no residual pigment in the cell pellets after the extraction and the resulting pigment was concentrated using rotary evaporator (STRIKE 300, Stereo Glass, Italy) at 50 °C, dried for 3 days at 40 °C followed by separation using column chromatography using petroleum ether: benzene: acetone (10:40:5). Fraction no. 4 and 5 contained the yellowish-orange pigment (flexirubin type of pigment) were pooled and characterized (Fig. 1).

2.3. Structural characterization of the pigment

The purified yellowish-orange pigment was determined for maximum wavelength, \( \lambda_{\text{max}} \) using UV–Vis spectrophotometer (Perkin Elmer Precisely Lambda 25) between 800 and 200 nm. The dried bacterial pellet (1 mg) was finely ground with 200 mg KBr (Scharlau), pressed under 0.414 bar followed by analysis using the FTIR spectrophotometer (Shimadzu, FTIR 8300) between 4000 and 400 cm\(^{-1}\). The powdered pigment was analyzed using the NMR spectroscopic analysis for \(^1H\) (400 MHz) and \(^{13}C\) (100 MHz) using Bruker Avance 400 NMR spectrometer. The chemical shifts were reported in parts per million relative to tetramethylsilane in deuterated dimethyl sulfoxide (DMSO) as solvent. The powdered pigment was dissolved in DMSO prior to analysis using the LC–MS (LCQ Deca XP) using electrospray ionization as the ion source.

2.4. Physico-chemical properties of the pigment

2.4.1. Pigment solubility

0.05 g of the pigment was added to 10 mL of water, organic solvents (acetone, benzene, chloroform, ethyl acetate, ethanol, methanol, petroleum ether, hexane and DMSO), alkali (Na\(_2\)CO\(_3\), NaOH) with stirring for 1 h and left for 30 min. Then the solution was filtered and absorption were recorded at \( \lambda_{\text{max}} \) to attain solubility of the pigment.

2.5. Stability of the pigment

2.5.1. Temperature

A 0.005 g of pigment solution was treated in a thermostatically controlled bath at 25, 50, 75 and 100 °C and the samples were held at each temperature for 0.5, 1, 1.5, 2, 2.5, 3 h, respectively, and then cooled immediately in an ice bath. The absorption spectra of the solutions was recorded at \( \lambda_{\text{max}} \).

2.5.2. Light

A 0.005 g of the pigment solution were held under sunlight, dark and under ultra-violet light for 5 days and the absorbance was determined at \( \lambda_{\text{max}} \).

2.6. Determination of color value of the pigment

The absorbance values of pigment solutions at 450 nm were adjusted to the range of 1.0–2.0 for color analysis. The values of \( L^a \), \( a^b \), and \( b^b \) were measured by a ColorFlex EZ meter with the CIELAB color system (Hunter Associates Laboratory, Inc., Virginia, USA). These values were then used to calculate chroma (\( C^\ast \)) and hue angle (\( h^\circ \)) values. \( L^a \) indicates lightness from 0 (black) to 100 (white). Positives and negatives in \( a^b \) represent red and green, respectively, whereas positives and negatives in \( b^b \) represent yellow and blue, respectively. Chroma values denote the saturation or purity of the color. Values close to the center at the same \( L^a \) value indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors. Hue angle values represent 0 for redness, 90 for yellowness, 180 for greenness, and 270 for blueness.

2.7. Statistical analysis

All experimental results were centered at using three parallel measurements of mean \pm standard deviation. Analysis of variance and probability tests were conducted to determine the degree of differences between the means obtained with \( P < 0.05 \) regarded as significant while \( P < 0.01 \) was considered very significant.

3. Results and discussion

3.1. Structural determination of the pigments

The orange pigment exhibited peaks at 450 nm when extracted in acetone (Fig. 2). The FTIR spectrum for the purified pigment is shown in Fig. 3. In the spectra of datiscetin, the peak at 3753 cm\(^{-1}\) is attributed to the hydroxyl stretching of absorbed water and the absorption band at 3454 cm\(^{-1}\) is due to the OH groups. The peaks between 1737 cm\(^{-1}\) and 1217 cm\(^{-1}\) corresponds to C–O stretchings. The shifts in carbonyl and the hydroxyl absorption spectra of the pigment can be related to strong chelation (Nagy et al., 1998;
Also the absorption bands at 893 to 526 cm\(^{-1}\) are due to asymmetric C–H stretching in alkyl hydrocarbons which corresponds to the flexirubin class of pigment (Bej, 2011).

The \(^1\)H NMR and \(^{13}\)C NMR spectrums of purified orange pigment are given in Fig. 4, in DMSO and its resonances have been completely assigned. Some flexirubin type pigment contains chlorine at ring A. The pigment showed certain structural peculiarities not found so far in other organisms. The chlorinated series was found in addition at B ring to the normal flexirubin.

The mass spectrum obtained from the LC–MS analysis exhibited a single major peak of molecular ion at \(m/z\) 159 \((m+1)\) 145 (Fig. 4), which is characteristic for the flexirubin class of pigment (12). The pigment exhibited an immediate color shift from characteristic orange to brown when flooded with 20% KOH and reverted to its initial color when flooded by an acidic solution once excess KOH was removed (Fautz and Reichenbach, 1980). The addition of 20% KOH changed the color of the pigment to dark orange and also broadened the peak, thus confirming that it is a flexirubin type of pigment (Weeks, 1981).

Flexirubin type of pigment contains \(\omega\)-phenyloctaenic acid chromophore esterified with resorcinol carrying two hydrocarbon chains. Their chemical structures are characterized by non-isoprenoid \(\omega\)-phenyl-substituted polyene carboxylic acids (Reichenbach and Kleinig, 1974). This basic chemical structure may be modified by variation of the length and branching of the hydrocarbon chains on the resorcinol and by the introduction of additional substituents on the \(\omega\)-phenyl ring, specifically methyl and chlorine, chlorinated counterparts are found in every flexirubin producing organism. In this way, a large variety of different flexirubin type pigments arise (Stafsnes and Bruheim, 2013).

Flexirubin has been proven to be located in the outer membrane of the bacteria and the concentration was found to be about 10 times as high as the phospholipid content in the outer membrane of ordinary Gram negative bacteria. Thus flexirubin functions as photoprotective compound or is involved in the
respiratory chain. Their presence and high concentration in the outer membrane suggests that they may have a structural function (Irschik and Reichenbach, 1978).

### 3.2. Physico-chemical properties of the pigment

#### 3.2.1. Solubility of the pigment

The results indicated that the pigment was insoluble in water and most of the common organic solvents (benzene, chloroform, ethyl acetate, ethanol, methanol, petroleum ether, hexane) and aqueous acid but soluble in alkaline solutions (Na₂CO₃ and NaOH) and slightly soluble in DMSO Table 1.

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<thead>
<tr>
<th>Tests</th>
<th>Response</th>
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<tr>
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<tr>
<td>Solubility in organic solvents</td>
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<td>Acetone</td>
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<td>Benzene, chloroform, ethyl acetate, ethanol, methanol, petroleum ether, hexane</td>
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<td>Solubility in 1 mol NaOH at 100 °C</td>
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<tr>
<td>DMSO</td>
<td>+</td>
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+++ Strong positive response.
++ Positive response.
– Negative response.

#### 3.3. Effect of temperature on the stability of the pigment

The absorbance of the pigment decreased with a loss of 0.027, while only 0.04 was lost from 25 to 50 °C. The color of the pigment was slightly changed to pale yellow and the results indicated that the pigment was slightly degraded at 100 °C.

#### 3.4. Effect of light on the stability of the pigment

The absorbance of the pigment slightly declined when exposed under sunlight and UV for 5 days, and remains same when exposed in dark. The color of the pigment has no obvious change and this indicated that light had less effect on the pigment (Fig. 5).

#### 3.5. Color characteristic of the pigment

Several models have been developed for the analysis of color, but the CIELab system is the one that nowadays presents a high acceptance since the color perception is uniform which means that the Euclidean distance between two colors corresponds approximately to the color difference perceived by the human eye (Hunt, 1991). The CIELab is an international standard for color.

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**Table 1**  
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**Fig. 4.** NMR and LCMS of orange pigment. LC–MS chromatogram showing the peak (ion selected at m/z 159) of the purified orange pigment with the retention time at 3.5 min.

**Fig. 5.** Effect of light on the stability of the pigment. Error bar represents standard deviation of the means (n=3).
measurement since 1976. \( L^* \) is the luminance component (from 0 to 100), and \( a^* \) (from green to red) and \( b^* \) (from blue to yellow) are the two chromatic components (Papadakis et al., 2000). The CIELab parameters of the pigment are presented in Table 2. \( L^* \) value was 3.58 and the pigment exhibited hue angle of 77.39 and chroma of 5.543 which represents yellow. As shown in Fig. 6 the pigment falls on the yellow region in two dimensional plot.

4. Conclusions

In this study, the pigment was isolated from *Chryseobacterium* UTM-3\(^3\). The pigment was purified and identified as flexirubin type of pigment. Further the physico-chemical properties were characterized and found the pigment was stable towards temperature and light. From the results of this study it is concluded that an interesting yellow pigment could be isolated and purified from *Chryseobacterium* sp. by submerged fermentation. However, large scale fermentation should be carried out for industrial applications. Further studies are required for this pigment particularly assessment of its potential in therapeutic uses and its applicability in food, textile industry etc.

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References

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