



The Identification of *Chromobacterium violaceum* From Soil Based on The Production of Violacein

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ABSTRACT

Chromobacterium violaceum is a purple coloured pathogenic bacterium mainly found in stagnant water and soil. Isolation and identification of this pathogenic bacteria are very important especially in biological science research and clinical field. It was easily identified by the production of purple coloured pigment violacein based on the solubility, colour reaction, spectrophotometry and growth temperature of *C. violaceum*. The density of *C. violaceum* found in 5 g of soil was much higher after rainy day which was about 7×10^7 located at $2^{\circ}59'09.5''\text{N}$ $101^{\circ}43'33.2''\text{E}$ and 1×10^7 located at $2^{\circ}58'56.3''\text{N}$ $101^{\circ}43'15.2''\text{E}$ compared to normal sunny day with only 6×10^5 and 9×10^4 in respectively at the same locations. The soils samples were collected 0-5 cm depth from the soil surface because of the facultative anaerobic where the *C. violaceum* from underground was more incline go to the top of soil since the concentration of oxygen was higher at the top.

Keywords: *Chromobacterium violaceum*, Violacein, Soil, Pathogenic.

INTRODUCTION

The purple coloured *Chromobacterium violaceum* is a Gram-negative pathogenic bacterium which can be found in most of the tropical country around the world mainly in stagnant water and soil (McGowan and Steinberg, 1995; Kothari *et al.*, 2017). It was reported negatively affect the plant growth and development (Lambers, 1980; Alstrom *et al.*, 1989; Schippers *et al.*, 1990; Loke and Halimi, 2010) and high fatal infection to human and animal (Richard, 1993; Siqueira *et al.*, 2005; Crosse *et al.*, 2006; Teoh *et al.*, 2006; Ajithdoss *et al.*, 2009; Hammerschmitt *et al.*, 2017).

The tropical weather in Malaysia offers a good environment for the growth of *C. violaceum*. The bacterium is commonly found in Malaysia soil. Soil is often contacted by people during their daily activities. Rapid identification of *C. violaceum* from soil is very important and useful for future study especially in agriculture, medical and health sciences.

According to Bergey's Manual of Systematic Bacteriology (2005) and Prokaryotes (2006), *C. violaceum* can be easily identified by the production of purple coloured pigment called violacein based on the solubility, colour reaction, spectrophotometry and growth temperature of *C. violaceum*. These few simple methods were much simpler and faster than conventional morphological and biochemical methods (Dworkin and Falkow,

2006; Kothari *et al.*, 2017). Besides that, it can overcome the disadvantages and limitations of 16S rRNA which are expensive, error from gene sequencing, incomplete database references, requires expensive laboratory equipment, infrastructure and skilled expertise (Jo *et al.*, 2016; Horton *et al.*, 2018; Wilson *et al.*, 2018; Muhamad *et al.*, 2020) and rarely able to identify up to species level due to having very high sequence similarities. (Petti, 2007; Lal *et al.*, 2011; Deurenberg *et al.*, 2017; Devanga Ragupathi *et al.*, 2017). Therefore, the aim of this work was to identify the *C. violaceum* from soil based on the production of violacein by *C. violaceum*.

MATERIALS AND METHODS

Preparation of growth medium

The medium used in this experiment were LB broth and LB agar (Luria and Burrous, 1955; Sambrook and Russel, 2001). The medium was determined by pH meter and adjusted to neutral by 0.1 M HCl and 0.1 M NaOH. After the pH was corrected, the medium was dispensed into test tubes by pipetting and capped. Tubes of media were sterilized in an autoclave (All American 25X, USA) for 20 minutes at 121°C. The LB broth in the tubes were let to cold down before use while LB agar in the tubes were cold down to 50°C for 5 minutes and the content of the tubes was poured into petri dishes and allowed to solidify.

Isolation of *Chromobacterium violaceum* from soil

The soils samples were collected 0-5 cm depth randomly from the soil surface at Universiti Putra Malaysia (UPM) during a sunny day and after rainy day for the microbial population count and isolation. The GPS coordinates for the sites were 2°58'56.3"N 101°43'15.2"E and 2°59'09.5"N 101°43'33.2"E. Six test tubes were labelled from 1 through 6 and 9 ml of saline were dispensed into each tube. Five g of soil was deposited into 100 ml bottle containing saline solution. The bottle was vortexed until all soil was well dispersed and a tenfold serial dilution was made from tube 1 to tube 6 by transferring 1 ml from each tube to achieve 1:10⁶ in the last tube. From the tubes, 1 ml from each tube was transferred to a dish of LB (Luria-Bertani) media (Sigma LB-Miller cat#L3027-1KG). The bacteria were spread on LB agar surface by using a sterile L-shaped glass rod on each dish and incubated for 30°C from 3 to 7 days. The dish contained purple pigment colonies were used for population count and identification after pure colony was obtained with several passages on LB media.

Identification

Violacein and *C. violaceum* can be identified by few methods according to Bergey's Manual of Systematic Bacteriology (2005) and Prokaryotes (2006). The purple pigment, violacein could be detected depending on its solubility in 96% ethanol instead of water. The colour reaction was observed in fresh ethanolic solution by adding a few drops of 25% (v/v) H₂SO₄ that gives a green colour and 10% (v/v) NaOH that first gives a green colour and slowly turn to reddish brown colour after several seconds. In spectrophotometrically (UV-Vis Spectrophotometer UV-1700, Kyoto, Japan), the fresh ethanolic solution will shows a minimum of 431 nm and maximum of 579 nm of absorption if violacein was found. Selective media have not been developed for *C. violaceum* but colonies on routine growth media are sufficiently used and can be recognized by their purple pigmentation. In order to differentiate the *C. violaceum* from other groups of violacein-producing bacteria, the isolated violacein-producing bacteria were grow in 37°C where only *C. violaceum* were able to grow.

RESULTS AND DISCUSSION

Bacteria isolated from 0-5 cm depth of soil surface at UPM was confirmed as *C. violaceum* which shown in (Fig. 1) after the identification based on Bergey's Manual of Systematic Bacteriology (2005) and Prokaryotes (2006). The purple pigment from the bacteria colonies was soluble in 96% ethanol. Colour reactions were observed where the fresh ethanolic solution turns to green colour after a few drops of 25% (v/v) H₂SO₄ and addition of

10% (v/v) NaOH has turned the fresh ethanolic solution to green colour and slowly turned to reddish brown colour after several seconds (**Fig. 2**). In spectrophotometer reading (UV-Vis Spectrophotometer UV-1700, Kyoto, Japan), the fresh ethanolic sample showed a minimum of 431 nm and maximum of 579 nm of absorption. These results indicated that the purple pigment produced by the bacteria was violacein. The purple pigment bacteria was able to grow at 37°C (**Table 1**) which proved that it was *C. violaceum* where no other violacein-producing bacteria able to grow at 37°C (**Table 2**).

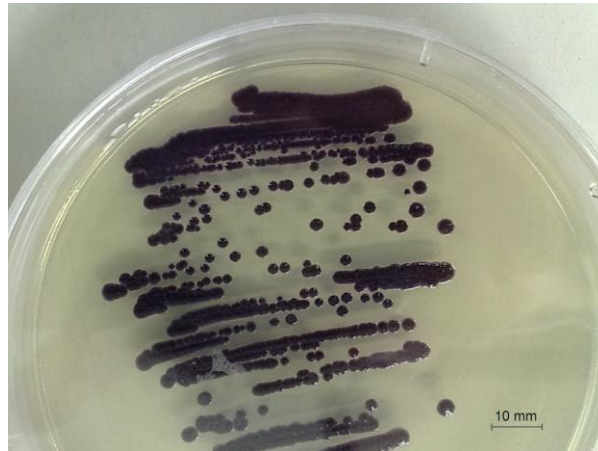


Fig. 1. Pure culture of *Chromobacterium violaceum*

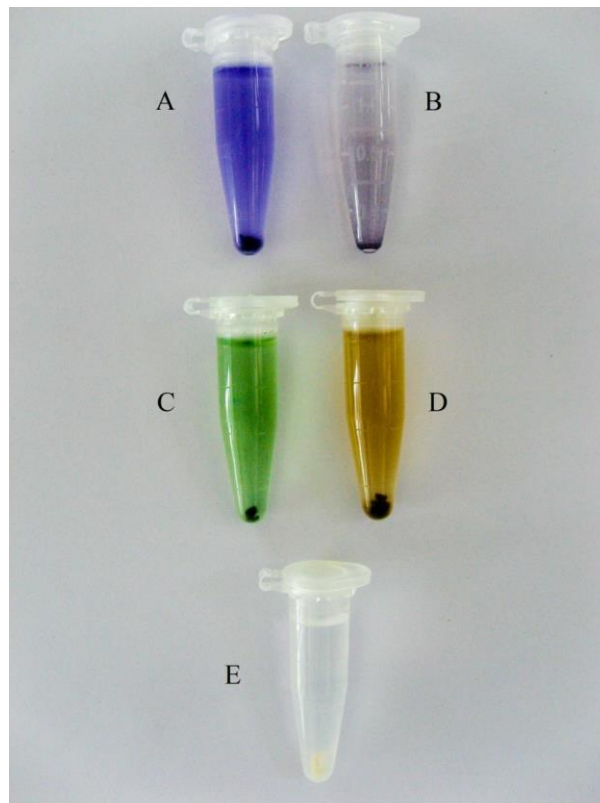


Fig. 2. Colour reaction of violacein. (a) Violacein from *C. violaceum* soluble in ethanol, (b) Violacein from *C. violaceum* not soluble in water, (c) Solution turns green after added 25% (v/v) H₂SO₄, (d) Solution turns brown after added 10% (v/v) NaOH, (e) *Rhizobium* sp. as control which did not have any colour reaction.

Table 1. *Chromobacterium violaceum* growth temperature.

Temperature	Colony
04°C	no
27°C	yes
32°C	yes
37°C	yes
40°C	yes

Table 2. Characteristics from violacein producer bacteria

Characteristics	<i>C. violaceum</i>	<i>Janthinobacterium</i>	<i>Iodobacter</i>
Able to grow at 4°C	no	yes	yes
Able to grow at 37°C	yes	no	no

(Source: Boone *et al.*, 2005; Dworkin and Falkow, 2006).

In comparison to 16S rRNA and other conventional methods such as morphological and biochemical characteristics, these few methods based on violacein production were more precise. It was focus only on the violacein pigments produce by the bacteria which were *Chromobacterium*, *Janthinobacterium* and *Iodobacter* with the advantages of uncomplicated procedures and inexpensive. (Boone *et al.*, 2005; Dworkin and Falkow, 2006; Kothari *et al.*, 2017).

In the population count, the density of *C. violaceum* in 5 g of soil was much higher after rainy day which was about 7×10^7 located at 2°59'09.5"N 101°43'33.2"E and 1×10^7 located at 2°58'56.3"N 101°43'15.2"E compared to normal sunny day with only 6×10^5 and 9×10^4 in respectively at the same locations. The reason why the density of *C. violaceum* from 0-5 cm of soil surface was higher after rainy day was supported by the moisture condition in soil (Iovieno and Bååth, 2008; Cruz-Paredes *et al.*, 2021) and the characteristic of facultative anaerobic (McGowan and Steinberg, 1995; Kothari *et al.*, 2017) where the *C. violaceum* from deeper soil was more incline to the top (Fig. 3) since the concentration of oxygen was much higher at the top and aerobic respiration is the most beneficial to *C. violaceum* (Voroney, 2007).

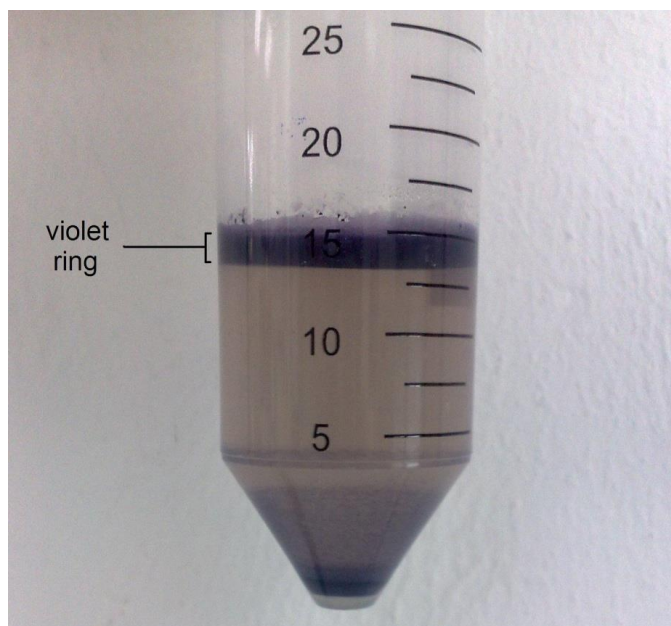


Fig. 3. *Chromobacterium violaceum* incline to the top to form a violet ring at the surface of LB broth.

CONCLUSION

Malaysia is a tropical country where the occurrence of *C. violaceum* was high and strong possibility of finding it in the soil and water. Isolation and identification of this pathogenic bacteria are very important for future research especially in clinical and biological science. This simple method of identification is an alternative to other conventional methods with a great advantage and uncomplicated which did not require costly reagents, infrastructure and equipments. Besides that, it has the potential to be developed as a chromogenic method for rapid identification.

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