



## IDENTIFICATION AND CHARACTERIZATION OF PIGMENTED BACTERIA ISOLATED FROM MALAYSIAN SEAWATER

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Article History: Received on 12<sup>th</sup> November 2019, Revised on 20<sup>th</sup> December 2019, Published on 30<sup>th</sup> December 2019

### Abstract

**Purpose of study:** Bacteria can naturally produce pigments that can be useful for various applications as they possess antimicrobial metabolites among other numerous benefits towards the human health. This study was carried out to identify the species of marine bacterial isolates PMA, PM3C1 and PM5C1 exhibiting yellow, orange and green colors respectively.

**Methodology:** The current study is using Polymerase Chain Reaction (PCR) amplification and sequence analysis of their 16S rRNA gene. The stability of pigments extracted from the bacterial samples was also analyzed against different temperature and light conditions.

**Main Findings:** Sequence alignment using BLAST revealed that the yellow, orange, and green-pigmented bacteria have 84% similarity with *Staphylococcus aureus*, 85% similarity with *Exiguobacterium profundum* and 95% similarity with *Pseudomonas aeruginosa* respectively. The green pigment showed major changes in color following exposure to sunlight and fluorescent light, and when incubated at 24°C and 50°C. Exposure to direct sunlight also results in the reduction of color for the yellow and orange extracts, while no effect was observed for both pigments under fluorescent light. Incubation at 50°C results in the reduction of the orange color, while the yellow pigment was observed to be unaffected suggesting its stability at high temperature.

**Implications:** Natural pigments production can provide many advantages including reduction of pollution generation, ease of disposal and other benefits to the human health.

**Keywords:** *Pigment, Marine Bacteria, Pigment Stability, Health.*

### INTRODUCTION

Pigments are used in many applications in the world such as the food industries that use pigments as food colorants, as well as in pharmaceutical and textile industries. These industries usually use synthetic pigments which are those chemically synthesized in the laboratories (Azlinah, 2016), rather than the natural pigments. Synthetic pigments have been associated with various risks such as the threat of allergenicity, toxicity, and pollution. The permitted synthetic pigments that are currently being used in industries have also been suggested to give potential health risks, carcinogenicity and toxicity that may cause damage to the human organs. Therefore, the demand for natural pigments by consumers especially as food colorants has increased due to their concern and awareness about health (Joshi et al., 2003). Furthermore, natural pigment production can provide many advantages including reduction of pollution generation, ease of disposal and other benefits to the human health. These natural pigments can be obtained from plants, animals, microorganisms and minerals (Rubia & Bhardwaj, 2016).

Many researches on pigments have started to focus on water-based natural pigments from the marine environment for the purpose of pharmacological, commercial and industrial applications due to their high biodiversity (Soliev, 2012). One of the promising sources of pigments is from marine microorganisms that include bacteria, which has the advantage over other sources since they are fast and easy to grow. Marine bacteria with commercial potential have been increasingly isolated, some of which are associated with invertebrate hosts (Kamarudin & Rehan, 2018). Bacteria can naturally produce various types of pigments including carotenoids, which are the naturally occurring red, yellow and orange pigments (Yadav & Prabha, 2014). Carotenoid pigments have been shown to have antioxidant activities, and have the potential to prevent cardiovascular diseases and cancer (Shindo & Misawa, 2014). Pyocyanin is another type of bacterial pigment that can be produced by certain strains of *Pseudomonas aeruginosa*, and have been shown to possess antimicrobial activity against Gram-negative and Gram-positive bacteria (Devnath et al., 2017).

The species of pigmented bacteria isolated from various locations including the marine environment can be genetically identified using the 16S ribosomal RNA gene (16S rRNA gene) sequence analysis (Janda & Abbott, 2007). This gene sequence has been widely used to describe novel organisms and pathogens (Clarridge, 2004). Previous studies have isolated pigment-producing bacteria from water and soil, followed by the identification and characterization of the bacteria. Among the isolated bacterial strains include the red-pigmented bacteria *Serratia marcescens*, violet pigment-

producing bacteria *Chromobacterium violaceum* and yellow-orange pigmented bacteria *Chryseobacterium* sp. (Ahmad et al., 2012).

The natural pigments are important in many types of industrial applications, and those that have high stability of colors against various factors are favored especially when used as a food colorant. The lower stability of natural bacterial pigments against environmental factors could pose restrictions and limitations to their utilization as a food colorant (Jenshi et al., 2011). The stability of some pigments has been reported to be affected by acidity, temperature and light (Dufossé, 2018). Hence, research on the stability of natural pigments can be useful for various applications. This study aimed to identify the species of three marine pigmented bacteria previously isolated from the marine environment that exhibits yellow, orange and green color. Crude pigments were also extracted from each bacteria and their stability against different temperature and light sources were analyzed.

## MATERIALS AND METHODS

### Sample Preparation and Staining

Three samples of pigmented bacteria previously isolated from the marine environment were used in this study, which is PMA, PM3C1 and PM5C1. These bacterial samples were visualized as yellow, orange and green colored colonies respectively on the LB agar media (Oxoid LTD, England). The samples were grown on LB agar for 24 hours at 30°C in the dark condition. A single colony of each sample was Gram-stained and their morphology observed under the microscope (Olympus CX21).

### Polymerase Chain Reaction Amplification of the 16S rRNA Gene

Genomic DNA was extracted from each bacterial sample using Promega Wizard® Genomic DNA Purification Kit (Promega Corp., USA) according to the manufacturer's instructions. The quantity and purity of extracted DNA were determined using NanoPhotometer® P-class (Implen, Germany) at the absorbance of 260nm and 280nm, prior to being used as a template in subsequent PCR reaction. Amplification of the 16S rRNA gene from each sample was carried out using the universal 16S rRNA gene bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3') (Srinivasan et al., 2015).

All PCR reactions were conducted in a 25 µL total volume consisting of 12.5 µL PCR Master Mix (Promega), 1.5 µL (0.7 µM) of each universal primer and 2 µL (74.4 to 81.3 ng/µL) of template DNA. The PCR conditions were carried out according to Fatin et al. (2018) with modifications, with initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 24 seconds, 55°C for 24 seconds, 72°C for 78 seconds, and a final extension step at 72°C for 10 minutes. The PCR reaction was carried out in the T100™ Thermal Cycler (Bio-Rad Laboratories, USA). The amplified products were analyzed on 1% (w/v) agarose gel (HyAgarose™) stained with ethidium bromide, and a 1 kb DNA ladder (Thermo Scientific™ GeneRuler™) was included as the standard DNA marker. The agarose gel was visualized under the UV light using the gel documentation system (UVITECH).

### DNA Sequencing and Analysis

The amplified 16S rRNA gene for each sample was sent to the 1st BASE Laboratory (Apical Scientific Sdn Bhd) for sequencing. The DNA sequences obtained for both forward and reverse 16S rRNA gene sequencing were observed using Chromas program version 2.5.1 and combined using the ClustalX program version 2.1 prior to alignment using the standard Basic Local Alignment Search Tool (BLASTn) of the NCBI database.

### Pigment Extraction and Analysis

Pigments were extracted from each sample according to the methods by Al-kazaz et al. (2014) with modifications, in which 1 ml of each bacterial strains were grown in 250 ml LB broth and incubated for 3 days at 30°C without light in a shaking incubator at 1600 rpm. The bacterial samples were centrifuged at 6000 rpm, 4°C for 4 minutes to get the cell-free extracts and transferred into a new 250 ml conical flask. Pigments that are trapped in the pellet were suspended with 3 ml methanol (ACI Labscan AR), followed by the addition of 150 ml chloroform (ChemAR®) into each conical flask. The supernatant produced after adding the chloroform was transferred into a new 250 ml conical flask. Each extract was placed in petri dishes and dried in the Protech Lab-Dryer (MyLab Scientific) overnight. The samples were then added with 7 ml of methanol before the samples were transferred into new 250 ml conical flasks. The extracted pigments were stored in the dark at -20°C.

### Effect of Light Exposure on Pigment Stability

The extracted pigments were exposed to different light conditions, which are under direct sunlight, under fluorescent light and without the presence of light (in the dark box). The samples were exposed to different conditions for 5 days, and any color changes were visualized and recorded. The extracts were also analyzed using UV-Visible Spectrophotometer (Cary 50 Conc) at the wavelength between 300 to 650 nm at room temperature.

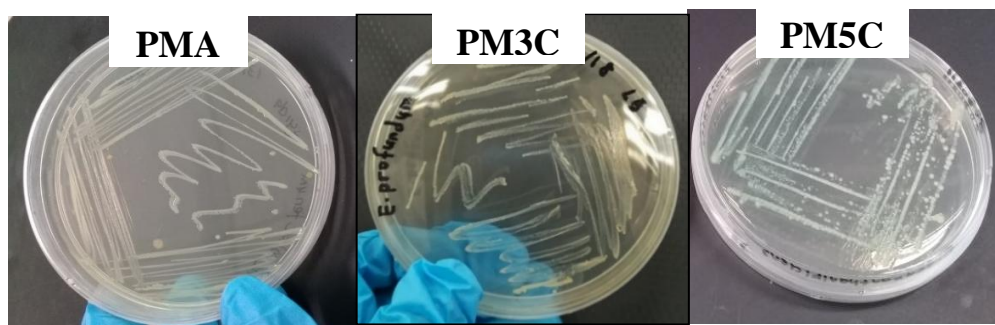
### Effect of Temperature on Pigment Stability

Samples were incubated at three different temperatures, which are 4°C, 24°C and 50°C for 5 days. The color changes of each extract were visualized and recorded. The extracts were also analyzed using UV-Visible Spectrophotometer (Cary 50 Conc) at the wavelength between 300 to 650 nm at room temperature.

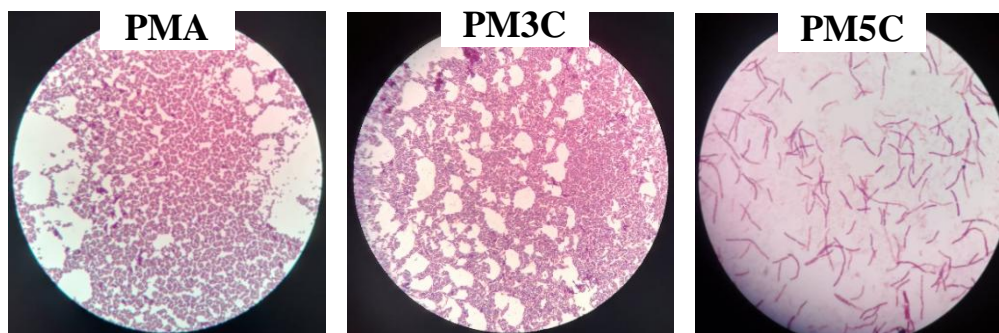
## RESULTS AND DISCUSSION

### Growth and Morphology of Bacteria

Three pigmented bacterial samples previously isolated from the marine seawater in Malaysia were morphologically analyzed by colony observation on plate media and under the microscope. The PMA strain was observed as light yellow colonies with the round shape of coccus or spheroid and found clusters that resemble a single cell or a bunch of grapes when observed under the microscope. The PM3C1 strain exhibited orange color on media and had a cocci shape, while PM5C1 isolate was observed as sticky, flat, opaque, consist of smaller colonies, producing green fluorescent pigment on the agar and have a rod shape under the microscope. Gram staining analysis indicated that the PMA (yellow bacteria) and PM3C1 (orange bacteria) isolates were Gram-positive bacteria, while sample PM5C1 (green) is a Gram-negative bacteria.



**Figure 1:** Morphology of the pigmented bacteria on plate media.



**Figure 2:** Morphology of the pigmented bacteria visualized under the microscope.

### Species Identification through 16S rRNA Gene Amplification and Sequence Alignment

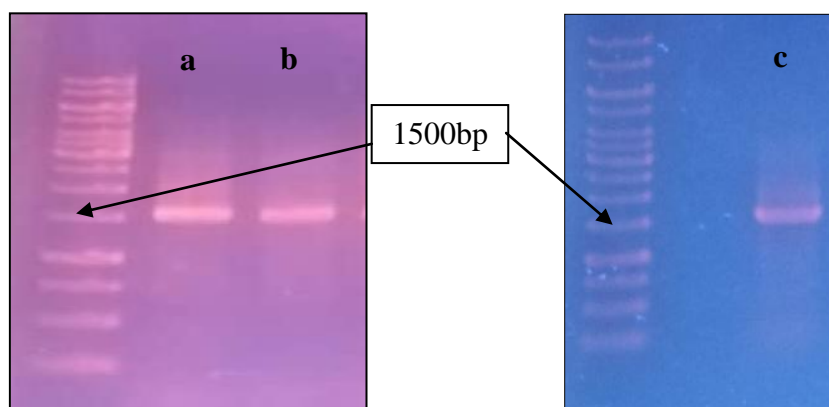
Total genomic DNA was extracted from each bacterial sample and analyzed for their concentration and purity using NanoPhotometer (Table 1). The DNA concentration obtained was in the range of 37.18 ng/μl to 40.64 ng/μl, while the DNA purity was analyzed at the absorbance of A260/A280, which was at the range of 1.5 to 2.1, indicating pure DNA obtained. The ratio between 1.7 and 2.0 is accepted as a high-quality DNA sample (Kheyrodin and Ghazvinian, 2012).

**Table 1:** The DNA concentration and purity of pigmented bacterial strains

Bacterial Isolates	Concentration (ng/μl)	Purity at A260/A280
PMA (yellow strain)	37.18	2.05
PM3C1 (orange strain)	37.47	1.93
PM5C1 (green strain)	40.64	1.56

The extracted DNA was used as templates in Polymerase Chain Reaction amplification of the 16S rRNA gene for species identification. Following amplification, DNA bands of approximately 1500 bp in size were observed on gel electrophoresis for all three samples (Figure 3), consistent with the expected size of the 16S rRNA gene for the universal primer pairs used in this study. The 16S rRNA gene is usually required and desirable when describing a new species (Clarridge, 2004).

Partial sequence of the 16S rRNA gene was obtained for each sample, with 1300 bp, 1163 bp and 1233 bp sequences for PMA, PM3C1 and PM5C1 strains respectively. Sequence alignment using BLASTn to the existing sequences in the NCBI database revealed that the sequence of PMA (yellow) strain has the closest relative with *Staphylococcus aureus* with 84% similarity, the PM3C1 (orange) strain with *Exiguobacterium profundum* with 85% similarity, while PM5C1 (green) strain have 95% similarities with *Pseudomonas aeruginosa* (Table 2).



**Figure 3:** PCR product of the 16S rRNA gene amplification. 1kb DNA Ladder was used as the standard marker. Samples include: a) PMA b) PM5C1 c) PM3C1.

**Table 2:** BLAST Analysis of 16S rRNA Gene Sequence of Pigmented Bacterial Strains

Bacterial Isolates	Closest Relative	% ID	Genbank No.	Accession
PMA (yellow)	<i>Staphylococcus aureus</i>	84%	MG230264.1	
PM3C1 (orange)	<i>Exiguobacterium profundum</i>	85%	KX999149.1	
PM5C1 (green)	<i>Pseudomonas aeruginosa</i>	95%	GU323371.1	

Note: ID – based on Identities score (Ident).

### Pigment Extraction

Crude pigments were extracted from the three bacterial samples using chloroform and methanol as solvents, yielding a range of colors in the cell-free extracts, in particular, yellow color for the *S. aureus* PMA sample, orange color for *Exiguobacterium sp.* PM3C1 strain and dark green color *P. aeruginosa* PM5C1. The bacteria *Staphylococcus aureus* and *Exiguobacterium sp.* have previously been reported to produce secondary metabolites that include the carotenoid pigments, which give them the yellow to orange colors (Liu et al., 2005; Manon et al. 2015). *Pseudomonas aeruginosa* is also known to produce a green pigment pyocyanin, which when combined with pyoverdine results in the characteristic of bright green color of *P. aeruginosa* (Devnath et al., 2017). Pyocyanin is a water-soluble bio-active compound produced by *P. aeruginosa* and has the capacity to exhibit antifungal activity and arrest the electron transport chain of the fungi (El-Fouly et al., 2015).

The solvent used for pigment extraction is also an important factor since it can determine the degree of affinity to the chemical composition of the pigment and have important roles in cell lysis. A study by Henriques et al. (2007) compared the differences between three solvents; ethanol, acetone, and methanol towards the extraction yields for chlorophyll pigment. Extraction using methanol produces the highest pigment yields compared to acetone and ethanol due to its hydrophilicity, which is considered to have a great extraction power that allows fast and effective pigment extraction (Henriques et al., 2007).

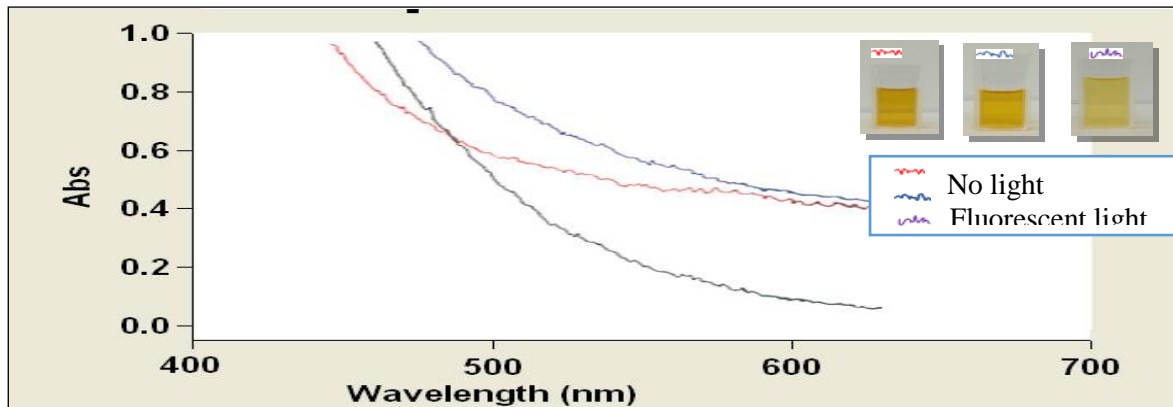
### The Effect of Light on Pigment Stability

The stability of crude pigments in the cell-free extracts for all three samples was analyzed by exposing them to different light conditions, which are direct sunlight, fluorescent light and without light exposure (in the dark). Visual examination of both yellow and orange color intensity in the extract samples showed no effect when exposed to the fluorescent light as compared to the dark condition. In contrast, exposure to direct sunlight after five days results in the reduction of color intensity of both yellow and orange samples as compared to the dark condition, suggesting that the pigments produced by *S. aureus* PMA strain and *Exiguobacterium sp.* PM3C1 could be affected by sunlight. Analysis of the absorbance using UV-visible spectroscopy at the range of 590- 670 nm for orange pigment (Figure 4) and at 400-500 nm for yellow pigment (result not shown) indicated that the absorbance reading of the cell-free extracts were the lowest when exposed to sunlight as compared to the fluorescent light and without light exposure.

Yip et al. (2014) had suggested that the carotenoid pigments can undergo isomerization and photodegradation when exposed to direct light. Light can lead and excite the formation of singlet oxygen, and can produce the excited state of

carotenoids when singlet oxygen reacts with carotenoids. The excited carotenoid state may cause degradation of the chemical pathway, and the trans-cis isomerization reactions promoted by light can result in pigment reduction (Yip et al., 2014).

The green-color extract from the *P. aeruginosa* PM5C1 strain was observed to exhibit color changes following five days of exposure to sunlight and fluorescent light as compared to the dark condition. Interestingly, exposure to the fluorescent light results in a change to brown color, while sunlight exposure changed the green-colored extract to orange-red color. The absorbance pattern of the green color extract was also found to be affected, consistent with its color changes under different light conditions. In particular, exposure to sunlight and fluorescent light showed higher absorbance value in the range of 600-700 nm compared to the dark incubation condition. These color changes may suggest that the green pigment is unstable when exposed to the fluorescent light and sunlight, and could be due to certain chemical reactions between light and pigments that cause pigment degradation.

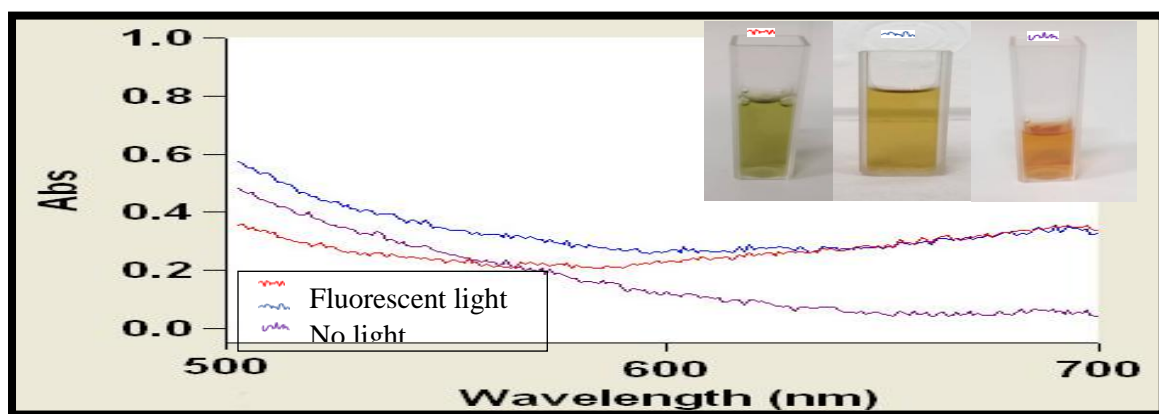


**Figure 5:** The effect of different light exposure (without light, fluorescent light, and sunlight) to the orange color intensity and absorbance spectrum of the cell-free extract of *Exiguobacterium* sp. PM3C1 strain.

#### The Effect of Temperature on Pigment Stability

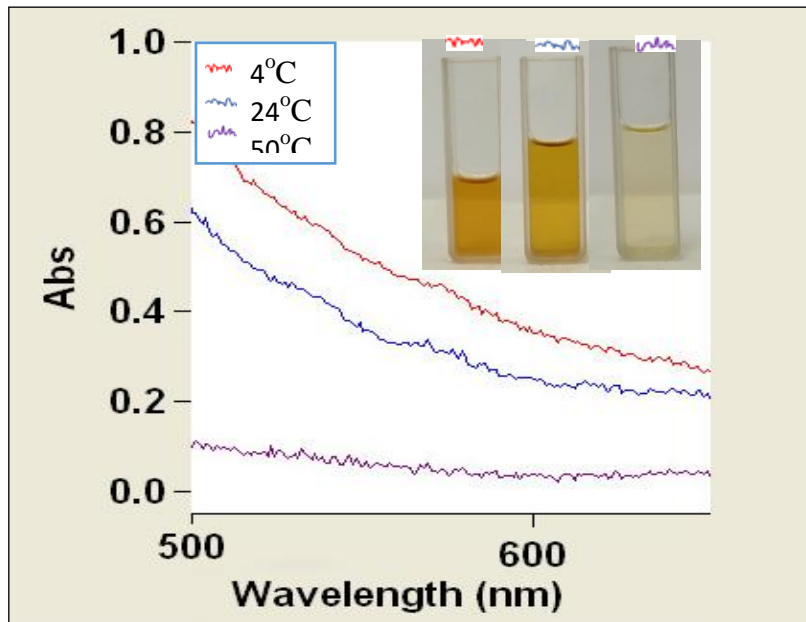
The crude pigment extracts were incubated at 4°C, 24°C, and 50°C for five days as a preliminary analysis of their stability towards the different range of temperatures. For the yellow extracts of *S. aureus* PMA strain, no changes of the yellow color intensity were observed by visual examination following incubation at 4°C, 24°C and 50°C. Analysis of the absorbance reading at 400-500 nm wavelength also showed no difference between the values (result not shown). This observation indicated that the crude pigments in the extracts were stable even at a high temperature, which is at 50°C. Further analysis of pigment stability in future studies that include its incubation at much higher temperatures could be beneficial for the potential use of the yellow pigment in various applications.

The orange color extract from *Exiguobacterium* sp. PM3C1 strain showed only a slight reduction of color and absorbance value at 500-600 nm wavelength when compared between incubation at 4°C and 24°C (Figure 7). However, incubation of the extract at 50°C reduced the orange color, suggesting that the orange pigment could be affected by high temperature. This reduction of colour may be caused by degradation during heat processing by isomerization, decarboxylation or cleavage, resulting in a gradual reduction of pigment intensity. It has been suggested that the double bonds in the carotenoid molecule can be broken and cause pigment degradation when exposed to high temperatures (Yip et al., 2014).

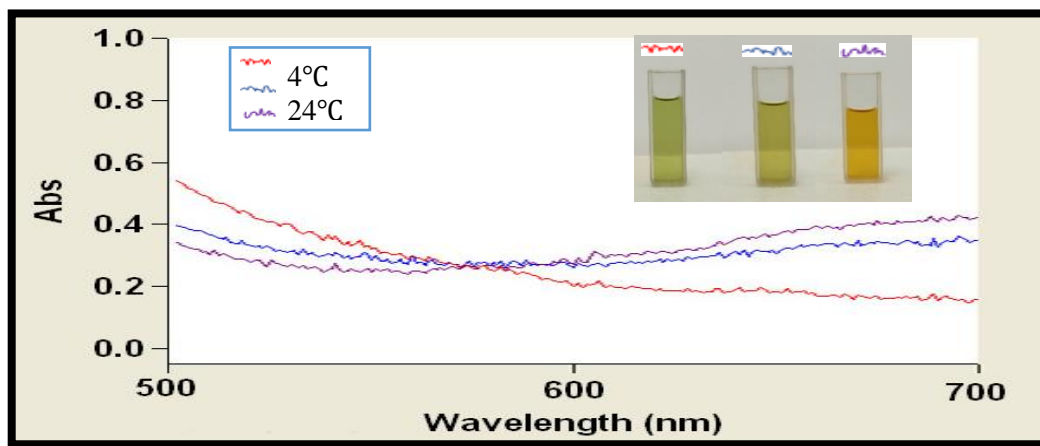


**Figure 6:** The effect of different light exposure (without light, fluorescent light, and sunlight) to the green color and absorbance spectrum of the cell-free extract of *Pseudomonas aeruginosa* PM5C1 strain.

For the green color extract from *Pseudomonas aeruginosa* PM5C1 strain, only minor change of color was observed through visual examination when the extract was incubated at 24°C as compared to 4°C. However, the green color changed to dark yellow upon incubation at 50°C, suggesting its instability at high temperatures. At 50°C treatment, the green extract showed relatively low absorption value at the wavelength ranging from 500-600 nm, and the highest at the range of 600-700 nm, consistent with the change of color from green to dark yellow. Reshmi et al. (2012) had reported that some chemical reaction may occur during heat treatment processing which can cause color changes. These interesting observations on the changes of green to other colors following different environmental stress exposure could be further analyzed for the color properties and potential applications.



**Figure 7:** The effect of different temperatures (4°C, 24°C and 50°C) to the orange color intensity and absorbance of the cell-free extract of *Exiguobacterium sp.* PM3C1 strain.



**Figure 8:** The effect of different temperatures (4°C, 24°C and 50°C) to the orange color intensity and absorbance of the cell-free extract of *Pseudomonas aeruginosa* PM5C1 strain.

## CONCLUSION

Three marine pigmented bacteria were characterized in this study, which is PMA, PM3C1 and PM5C1 exhibiting yellow, orange and green colors respectively. Sequence alignment of the 16S rRNA gene identified the strains to their closest relative of *Staphylococcus aureus*, *Exiguobacterium sp.* and *Pseudomonas aeruginosa*. Preliminary analysis of crude pigments extracted from the three bacterial samples suggested that the yellow pigments of *Staphylococcus aureus* PMA is stable against various incubation temperature, with minor reduction of color upon sunlight exposure. The orange pigments of *Exiguobacterium sp.* PM3C1 is affected by the high temperature of 50°C and sunlight exposure. The green extract from *Pseudomonas aeruginosa* PM5C1 showed major changes of color at 24°C and 50°C incubation as compared to 4°C, and under sunlight and fluorescent light exposure as compared to the dark condition.

## ACKNOWLEDGMENTS

The authors acknowledged all members of Food Biotechnology program, Faculty of Science and Technology (FST), Universiti Sains Islam Malaysia (USIM), Nilai, Negeri Sembilan. Special thanks to Ms. Sarina Irma Saidon, Mr. Muhammad Izzat Redzuan, and Mrs. Siti Nabilah Mohd Rusly for all the technical assistance. This research was funded by the Fundamental Research Grant Scheme (FRGS) – Ref: USIM/FRGS/FST/32/51515.

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