CHAPTER FIVE

SPECTROPHOTOMETRIC ANALYSIS AND ANTIBACTERIAL ACTIVITY SCREENING OF MARINE PIGMENTED BACTERIAL EXTRACT

5.1 Introduction

The marine environment is complex and dynamic, with extreme variations in pressure, salinity and temperature, which can lead to the development of unique metabolic and physiologic capabilities of the inhabiting microorganisms for their survival in these conditions (Balraj *et al*., 2014). This may also result in the production of new metabolites by the marine microorganisms that differ from those originated from terrestrial environment, as part of their unique metabolism (Fitri *et al.,* 2017). Some microbial communities also establish symbiotic relationship with other organisms in the marine ecosystem to adapt and survive in the highly competitive environment (Jensen and Fenical, 1994). A wide variety of unique secondary metabolites associated with this symbiotic relationship have also been discovered with significant biological properties (Zhang *et al*., 2015).

Sea cucumbers are among the marine invertebrates that offer desirable nutrient-rich habitat for the proliferation of microorganisms (Velho-Pereira and Furtado, 2012). Sea cucumbers are also well-known for their secondary metabolites and pigments such as carotenoids (Pangestuti and Arifin, 2018). Biosynthesis of these compounds may also be contributed by the symbiotic or associated microorganisms, or derive from a diet of

microorganisms (Farjami *et al.,* 2013). Thus, marine invertebrate-associated microorganisms are considered important sources of bioactive compounds (Wibowo *et al.,* 2019).

During the past two decades, research on marine bacteria has highlighted the tremendous potential of these microorganisms as a source of new secondary metabolites. This includes a wide variety of microbial pigments such as carotene, melanin, phenazine, pyrrole, violacein, and quinones (Velmurugan *et al.,* 2020). Pigments produced by marine bacteria are considered as the bioproducts with great potential for various industrial applications (Usman, 2017). For example, carotenoid pigments have been proven to possess strong antioxidative activities, and have been used commercially as food colorants, animal feed supplement, as well as for nutraceuticals, cosmetic and pharmaceutical purposes (Sasidharan *et al.* 2013). In addition, the phenazine-based pyocyanin pigment that can be produced by *Pseudomonas* sp. are also of particular interest since they have antitumor bioactivity, due to their capability to generate reactive oxygen species (Hassani *et al.,* 2012). Pyocyanin can also be used as biosensors, with the potential to be applied in the agricultural, medicinal, and environmental fields (Priyaja, 2013).

Several marine bacterial pigments have demonstrated various biological activities such as antimicrobial, anticancer, and immunosuppressive activities (Soliev *et al.*, 2011). Bacterial pigments were also shown to display strong and broad range of antibiotic activities (Holmstrom *et al*., 2002; Bruhn *et al*., 2007). Therefore, pigmented bacteria from the marine sources have become an important target for the discovery of new bioactive compounds, which can be utilized to overcome the antimicrobial

resistance crisis. Antimicrobial resistance (AMR) is a critical global threat which decreases the opportunities for the treatment of infectious diseases caused by viruses, bacteria, and fungi (Guo *et al.,* 2015).

One of the primary diagnostic tools for identification of microbial pigments especially carotenoids, is to study the spectrum of the organic solvent extract (Rodriguez-Amaya et al., 1999). For example, analysis of the pigments' UV-visible absorption spectra may provide an insight into the pigment characteristics, since it may assist in determining the pigment structure (Godinho and Bhosle, 2008). In this chapter, the crude methanolic extract from pigmented bacterial strains isolated from sea cucumbers and surroundings were analysed, and their antibacterial activity were screened against Gram-positive and Gram-negative bacterial representatives.

5.2 Materials and Methods

5.2.1 Production and Extraction of Pigmented Bacteria

Distinct colonies of bacteria were selected based on the pigmentation and sub-cultured onto fresh Nutrient agar media (Merck Darmstadt, Germany). A loopful of bacterial isolates were further grown in 5 ml Nutrient Broth (Merck Darmstadt, Germany) at 180 rpm in 37°C for 24 h using rotary shaker. Each pigmented culture was transferred into 50 ml Beckman centrifuge bottle and centrifuged at 10,000 rpm for 10 mins. Then, the resulting supernatant were collected and filtered through Whatman filter paper. Following that, the filtrate was concentrated using a rotary evaporator and 5 ml of methanol was added for further extraction at the ratio of (1:1, v/v). The methanolic

extract were reconcentrated using rotary evaporator to obtain minimal volume of extract.

5.2.2 Analysis of Pigment Extract using UV-Visible Spectrophotometer

The extracted pigments were analysed using UV-Visible spectrophotometer (UV-Visible 3600, Shimadzu Spectrophotometer, Japan) to determine the pigment profile between the range of 200 nm to 800 nm and was read in triplicate. For each sample, 100 µl was used by keeping methanol as reference blank.

5.2.3 Preparation of Test Bacteria for Antibacterial Activity

A colony of each bacterial isolate was inoculated in Mueller-Hinton broth (MHB, Oxoid, USA) at 37°C with gentle agitation overnight. The bacterial growth was monitored by measuring the optical density at 600 nm using BioPhotometer (Eppendorf AG, Hamburg, Germany). The concentration of bacterial cultures were determined by referring to MacFarland Standard Chart as shown in Appendix A. All bacterial cultures were further diluted to 0.5 MacFarland with the same medium.

5.2.4 Screening of the Bacterial Isolates for Antibacterial Activity

Antibacterial activity of the pigmented bacterial strains' extracts were screened against five pathogenic bacterial type strains that were obtained from the collection of Microbiology Lab, Faculty of Science and Technology, Islamic Science University of Malaysia. The selected test bacteria include two representatives of Gram-positive bacteria, which are *Staphylococcus aureus* and *Streptococcus faecalis;* and three representatives of Gram-negative bacteria, which are *Shigella* sp.*, Salmonella typhi* and *Escherichia coli.*

First, 100 µl of test bacteria was spread on to Mueller-Hinton agar plate (MHA, Oxoid, USA). Sterile paper disk containing 10 µl of the bacterial isolates were placed on the respective agar surface. The plates were then incubated at 30˚C for 24 h. Bacterial isolates that have the ability to kill or inhibit the test bacteria growth will display clear transparent zones around the spots of inoculation. The zone of inhibition was measured, and antibacterial activity was studied. In this assay, isolates were regarded as being antibacterial if inhibition zones formed is greater than 9 mm around the paper disk (Burgess *et al*., 2003). Three replicates were carried out for each antibacterial activity test. Methanol was used as the negative control, while and the Streptomycin sulphate solution was used as the positive control.

5.3 Results and Discussion

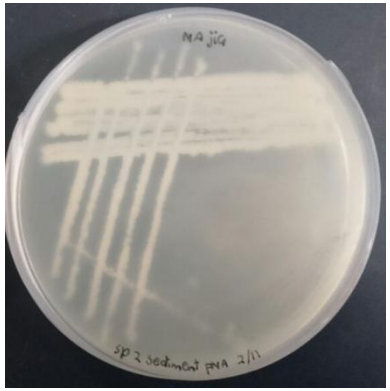
5.3.1 Morphological Observation of Pigmented Bacteria

A total of seven out of 58 bacterial isolates were observed to display vivid and bright coloration when grown on solid growth media, indicating the bacterial ability to produce pigments. All seven pigmented bacterial samples were isolated from sea cucumbers and sediment samples collected from Manukan Island, Sabah (Table 5.1). However, no pigmented bacteria were isolated from Pangkor Island's sea cucumber samples.

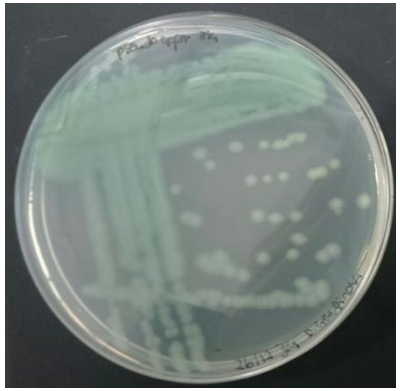
The seven pigmented bacterial samples were those isolated from cuticles, tentacles and polian vesicles of *Holothuria atra* (PMC and PMD), cuticle of *Holothuria hilla* (PME) and from the sediment collected surrounding *Bohadscia vitiensis* (PMB) (Table 5.1). Six bacterial samples were isolated from the external parts of sea cucumbers including one isolate from sediment, while only one pigmented bacterial strain was isolated from the internal part of a sea cucumber, specifically from Polian vesicle of *Holothuria atra* (PMD). These seven bacterial colonies displayed certain colours on the media which are yellow, green, pink and orange (Figure 5.1). The yellow pigmented bacteria include *Staphylococcus pasteuri* (PMBS3), *Paracoccus zeaxanthinifaciens* (PMCC2), *Microbacterium oxydans* (PMCT1) and *Staphylococcus saprophyticus* (PMDP). The sample *Acinetobacter pittii* (PMDC2) colonies displayed pink colour, *Pseudomonas aeruginosa* (PMCC1) displayed green colour, while *Exiguobacterium profundum* (PMEC2) showed orange-coloured colonies.

Table 5.1. List of pigmented marine bacteria associated with sea cucumber samples.

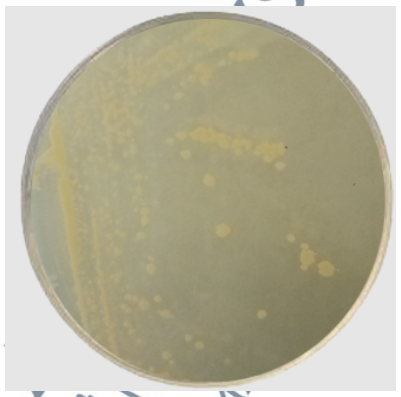
Bacteria ID	Bacterial Species	Color	Sources of isolates
PMBS3	*Staphylococcus pasteuri*	Yellow	Sediment surrounding *Bohadscia vitiensis* (PMB)
PMCC1	*Pseudomonas aeruginosa*	Green	Cuticle of *Holothuria atra* (PMC)
PMCC2	*Paracoccus zeaxanthinifaciens*	Yellow	Cuticle of *Holothuria atra* (PMC)
PMCT1	*Microbacterium oxydans*	Yellow	Tentacle of *Holothuria atra* (PMC)
PMDC2	*Acinetobacter pittii*	Pink	Cuticle of *Holothuria atra* (PMD)
PMDP	*Staphylococcus saprophyticus*	Yellow	Polian vesicle of *Holothuria atra* (PMD)
PMEC2	*Exiguobacterium profundum*	Orange	Cuticle of *Holothuria hilla* (PME)



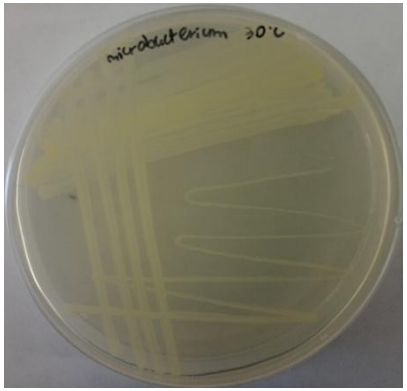
Staphylococcus pasteuri PMBS3



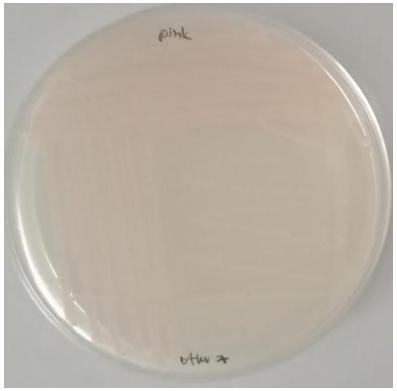
Pseudomonas aeruginosa PMCC1



Paracoccus zeaxanthinifaciens PMCC2

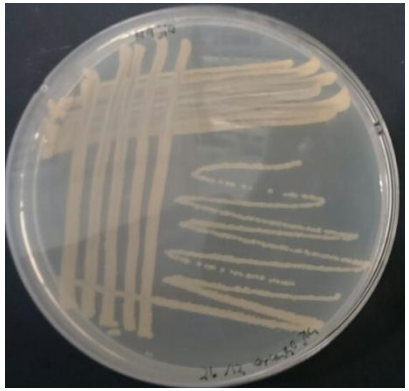


Microbacterium oxydans PMCT1



Acinetobacter pittii PMDC2

Staphylococcus saprophyticus PMDP



Exiguobacterium profundum PMEC2

Figure 5.1. Pigmented bacteria isolated from sea cucumbers that display vivid coloration on media.

Colours observed from each bacterial sample are similar with previously recorded colours of each pigmented bacterial species. For example, strains from *Pseudomonas aeruginosa* species are known to produce pigments from the phenazine compounds and the most widely studied phenazine is the water soluble blue-green pyocyanin (5-N-methyl-1-hydroxy-phenazine). Many phenazine-producing bacteria are also commonly found associated with host organisms (Pierson & Pierson, 2010), consistent with the green coloured *Pseudomonas aeruginosa* (PMCC1) that was isolated from cuticle of *Holothuria atra* (PMC) in this study.

One of the yellow-coloured bacteria is *Microbacterium oxydans* PMCT1 isolated from the tentacle of *Holothuria atra* (PMC), which shows similar coloured colonies with a marine isolate of the same bacterial species shown in a previous study (Leiva *et al.,* 2015). It has been reported that *Microbacterium oxydans* is one of the Gram-positive bacteria that can produce carotenoid pigments (Aruldass *et al.,* 2018), which also have an antioxidant ability (Meddeb-Mouelhi *et al.,* 2016).

Apart from that, two bacterial isolates from the *Staphylococcus* genus also exhibited yellow colour, which are *S. pasteuri* PMBS3 isolated from sediments surrounding *Bohadschia vitiensis* (PMB), and *S. saprophyticus* PMDP from Polian vesicle of *Holothuria atra* (PMD). Even though many staphylococci are known to display non-pigmented colonies, there are species that have the characteristic of yellow-orange colonies or exhibiting yellowish pigmentation (Becker *et al.,* 2014). *S. pasteuri* is among the yellow staphylococci (Savini *et al.,* 2009), which explains the observation of yellow colonies for *S. pasteuri* PMBS3.

For *S. saprophyticus*, the colony pigments have been reported to be variable and most strains are not pigmented, while some may have a slight yellow tint which increases in intensity with age (Health Protection Agency, 2007). In this study, *S. saprophyticus* strain PMDP was observed to display yellow colonies. However, two other *S. saprophyticus* isolates which are PMDL and PMDT1 strains only displayed white colour on the LB and NA growth media, suggesting that the two strains are non-pigmented. One possibility of the pigment compound responsible for the yellow colour of *S. pasteuri* (PMBS3) and *S. saprophyticus* (PMDP) is staphyloxanthin, since this pigment has widely been reported to be produced by staphylococci including *S. aureus* (Mohammadi, 2012; Alshamaa & Issam, 2017) and *S. gallinarum* (Barretto & Vootla, 2018). However, further studies are needed to identify the yellow pigment compound.

The bacteria *Paracoccus zeaxanthinifaciens* PMCC2 isolated from *Holothuria atra* (PMC) cuticle is another sample that exhibited yellow-coloured colonies. This corresponds to previous studies on marine isolates of *P. zeaxanthinifaciens* with the ability to produce a yellow-coloured carotenoid pigment zeaxanthin (Raguénes *et al.,* 2004; Berry *et al.,* 2003). Zeaxanthin is mostly produced by cyanobacteria to provide protection against photooxidation (Sajilata *et al.,* 2008). Interestingly, another isolate of *P. zeaxanthinifaciens* PMDC3 displayed only white colonies, which may suggest that there is a strain variation of this species with regard to their ability to produce the yellow pigment.

Similarly, pink coloured colonies were observed for *Acinetobacter pittii* PMDC2 isolated from *H. atra* PMD cuticle, but the pink colour was not observed for another sample of *Acinetobacter pittii* PMDS1 isolated from sediments surrounding the

H. atra PMD. Bacteria from the genus *Acinetobacter* have long been known to be non-pigmented (Wong *et al*., 2017), and there is lack of information on pigments produced by *Acinetobacter* sp. However, a recent study by Liu *et al.* (2020) found the presence of an *Acinetobacter pittii* strain G61 isolated from the gut of golden scallops collected from Shen'ao Bay of Guangdong Province, China that exhibit orange pigmentation, which are likely to be carotenoids. Hence, strains of *Acinetobacter pittii* may also be varied in terms of their pigment producing ability, as was shown in this study for samples PMDC2 and PMDS1.

Other than that, another bacterial isolate *Exiguobacterium profundum* PMEC2 was observed to exhibit orange-coloured colonies on growth media. *E. profundum* has previously been known as moderately thermophilic and halotolerant bacteria, and morphologically characterised as creamy or orange under anaerobic or aerobic conditions (Crapart *et al*., 2007). A study by Sasidharan *et al.* (2013) reported that *E. profundum* isolated from air and soil samples also showed deep orange pigmentation when cultured on NA medium, which are most likely to be carotenoid pigments. Similarly, an *Exiguobacterium profundum* strain BC2-11 with the ability to produce orange-coloured metabolite was also isolated from the terrestrial soil samples of steel mills (Manon *et al.,* 2015). An *Exiguobacterium sp.* isolated from the marine samples of India Peninsular Region also exhibited orange-coloured pigments that likely belong to the carotenoid family (Balraj *et al*., 2014).

5.3.2 UV-visible Absorption Spectra of Crude Pigment Extracts

Crude pigments of the seven pigmented bacterial samples were extracted using methanol as a solvent. Methanol was chosen because it is a hydrophilic solvent that were previously shown to be suitable for extraction of many types of pigments, especially polar pigments such as carotenoids (Henriques *et al.*, 2007). The crude pigments obtained were further characterized using UV-visible spectrophotometer in the visible range between the wavelengths of approximately 200-800 nm. From the seven pigmented bacterial samples, the methanolic extracts of five isolates showed results when analysed using the UV-visible spectrophotometry (Table 5.3; Figure 5.2), while two other samples failed to produce any reading, which are those extracted from *Paracoccus zeaxanthinifaciens* PMCC2 and *Staphylococcus pasteuri* (PMBS3).

Table 5.2. Absorption peaks of methanolic extracts from pigmented bacteria

Isolate Number	Bacterial Species	Colour of pigment	Absorption maxima (λ_{max}) (nm)
PMCC1	*P. aeruginosa*	Green	2.69 (207)
PMCT1	*M. oxydans*	Yellow	0.25 (467)
PMDC2	*A. pittii*	Pink	0.13 (402)
PMEC2	*E. profundum*	Orange	0.20 (563)
PMDP	*S. saprophyticus*	Yellow	0.48 (460)

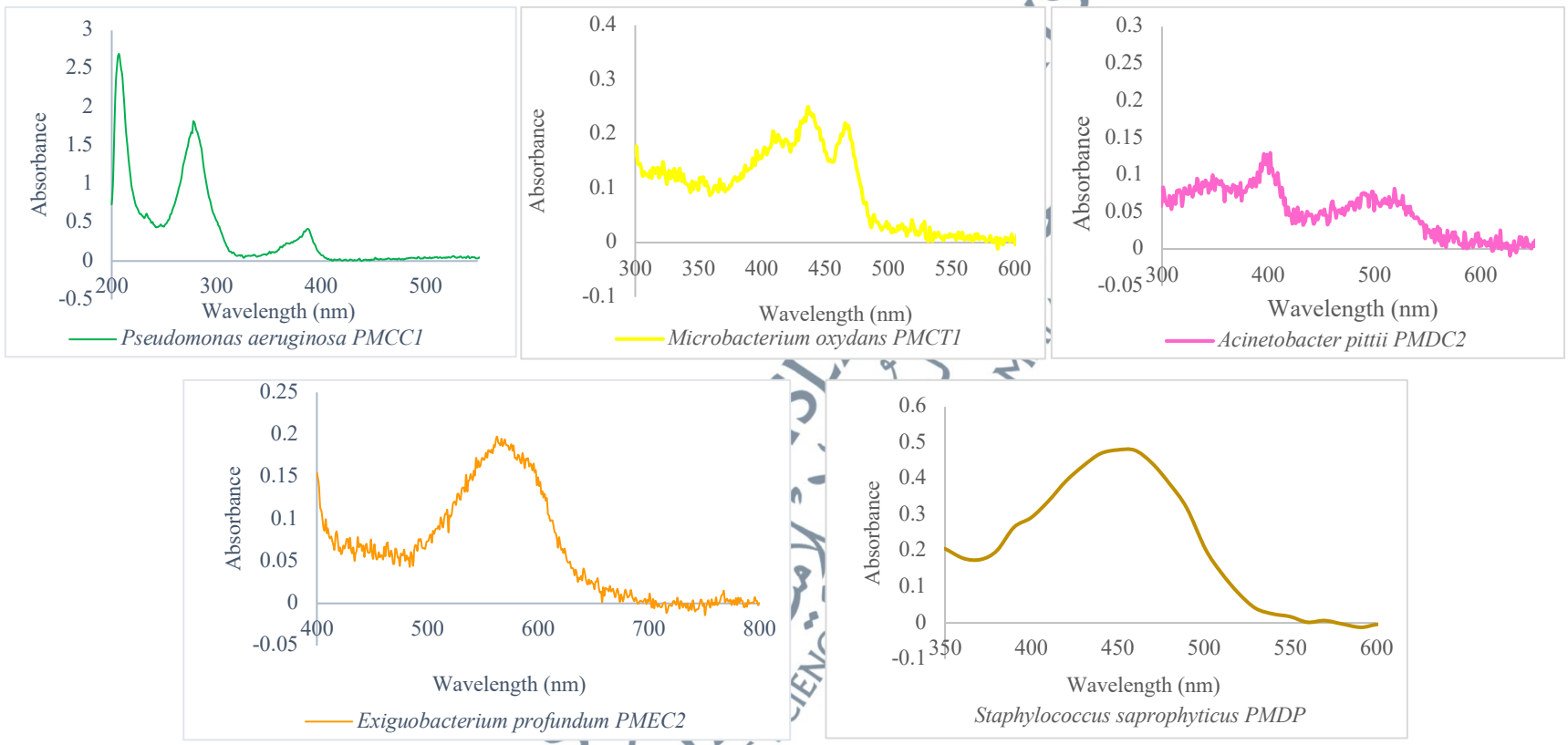


Figure 5.2. UV absorption spectra of the extracted pigment for samples PMCC1, PMCT1, PMDC2, PMEC2 and PMDP

Crude pigment extract from *P. aeruginosa* PMCC1 exhibited absorption maxima at three peaks, which are 207, 278 and 387 nm (Figure 5.2). This absorbance spectrum corresponds to a previous finding by Ohfuji *et al*. (2004) for pyocyanin methanolic extract especially at the range of 200 to 400 nm. The authors reported five characteristic peaks of pyocyanin methanolic extract of 201, 238, 318, 711 and 887 nm. The result is also comparable to the one reported by El-Fouly *et al.* (2015), in which the characteristic peaks of pyocyanin were observed at 316, 367 and 700 nm when dissolved in methanol. Furthermore, Darwesh *et al.* (2019) showed four peaks of pyocyanin after complete purification and dissolving in 15% methanol, which showed maximum wavelength at 280, 325, 495 and 710 nm. These corresponding absorbance spectra suggested that the green pigment extracted from *P. aeruginosa* PMCC1 could also be pyocyanin or other phenazine-related pigment.

For *Microbacterium oxydans* PMCT1, maximum absorption of its yellow methanolic extract were detected at peaks corresponding to 437, 441 and 466 nm. This absorbance spectrum correlates with Meddeb-Mouelhi *et al.* (2016) for purified yellow pigment from a *Microbacterium oxydans* strain isolated from a decomposing tree stump that exhibited peaks at 415, 435, and 465 nm. In addition, Ojha *et al.* (2018) reported that the methanolic pigment extract from a novel *Microbacterium paraoxydans* isolated from soil sample exhibited peaks at 407, 436 and 466 nm. This range of 400 to 500 nm is the region well-conserved for β-carotene-related molecules (Meddeb-Mouelhi *et al.,* 2016; Godinho & Bhosle 2008).

The methanolic extract of pink bacterial isolate *Acinetobacter pittii* PMDS1 also showed a reading when measured from 300 nm to 600 nm. The highest absorbance

value was observed at 402 nm, which suggests that the pigment is in the carotenoid group. This is because one of the characteristic features of carotenoids is light absorption in the high-energy zone of the visible region, which is in the range of 400–500 nm due to the long conjugated double bond system (Zaghdoudi *et al.,* 2017). However, this absorbance value of 402 nm for the pink extract of *Acinetobacter pittii* PMDS1 is slightly different than the maximum value of 478 nm previously shown for an orange carotenoid pigment extracted from *Acinetobacter pittii* G61, extracted using acetone (Liu *et al.,* 2020). This difference in maximum absorbance value could be due to the different types of carotenoids produced by the two strains.

The UV-visible spectrophotometric analysis of the crude orange pigment extracted from *Exiguobacterium profundum* PMEC2 colonies showed the highest absorbance peak at 563.0, when analysed between 400 to 800 nm. This absorbance peak is different compared to the study by Balraj *et al.* (2014) that obtained the highest peak at 450 nm for the orange pigment purified from a marine *Exiguobacterium* sp., or the usual peaks reported for carotenoid pigment groups. One possibility is that this may indicate different types of pigments produced by the two strains of *Exiguobacterium* sp. Furthermore, there are at least 700 carotenoids characterized from two of the known carotenoid biosynthetic pathway in many organisms (Godinho and Bhosle, 2008), hence different types of carotenoid pigments may show absorption at different wavelengths. Even though carotenoid pigments were usually reported to exhibit peaks between 400–500 nm, however some pigments may absorb light at higher wavelength. For example, the absorbance curve of purified β-carotene globules from algae were observed at the range of 350–550 nm (Kleinegris *et al.,* 2010). Besides, this may also indicate other types of pigments produced by *E. profundum* PMEC2.

For *Staphylococcus saprophyticus* PMDP isolated from Polian vesicle of *Holothuria atra* (PMD), analysis of the yellow pigment extract showed an absorption maximum at 469 nm. This is comparable to a previous study by Alshamaa & Issam (2017) that reported a peak at 460 nm for the orange-carotenoid pigments extracted from *Staphylococcus aureus,* which is likely to be a golden triterpenoid carotenoid staphyloxanthin. In fact, an orange pigmented *Staphylococcus kloosii* have also been isolated from *H. leucospilota* from Teluk Nipah, Pangkor Island and its methanolic extract showed maximum absorption at 426, 445 and 473 nm (Kamarudin *et al*., 2013).

Other researchers also reported similar absorption spectrum of purified staphyloxanthin, such as those extracted from *Staphylococcus carnosus* showing peaks at 463 and 490 nm (Pelz *et al*., 2005), while from a strain of *S. aureus* exhibiting a peak at 478 nm (Clauditz *et al*., 2006). The pigment staphyloxanthin has been extensively studied in *S. aureus* and some other *Staphylococcus sp.* (Mohammadi, 2012; Alshamaa & Issam, 2017; Barretto & Vootla, 2018), suggesting that *Staphylococcus saprophyticus* PMDP isolate may also produce staphyloxanthin that is responsible for the yellow colour. However, to date, there are no report from previous studies on the characteristics of yellow pigment produced by *S. saprophyticus*.

Two pigmented bacterial isolates did not show readings from UV-visible spectrophotometry analyses, which are *P. zeaxanthinifaciens* PMCC2 and *S. pasteuri* PMBS3. One possibility could be that production of pigments from these two strains need to be enhanced prior to extraction or purification to obtain sufficient amounts of pigments to be read through the UV-visible spectroscopy. The pigments produced by these bacterial strains may also be subjected to oxidation, which decrease the colour.

Clauditz *et al*. (2006) elucidated that the absorption maxima of purified staphyloxanthin from *S. aureus* showed significant reduction in absorbance readings at 478 nm when subjected to oxidation by hydroxyl radicals generated in a Fenton reaction. Apart from that, solvent used for extraction may not be suitable to obtain high amounts of pigment. For example, some strains of *P. zeaxanthinifaciens* are previously known to produce zeaxanthin (Raguénes *et al.,* 2004; Berry *et al.,* 2003), and this pigment was shown to be most soluble in ethanol instead of methanol in the study by Li and Engelberth (2018). Hence, optimization of the conditions of pigment production and extraction need to be performed to analyse these pigments.

Overall, the UV-visible spectrophotometry analysis suggested that the yellow pigment produced by *Staphylococcus saprophyticus* PMDP and *Microbacterium oxydans* PMCT1, as well as the pink pigment from *Acinetobacter pittii* PMDS1 could be from the carotenoid groups, while the green pigment from *P. aeruginosa* PMCC1 is likely to be phenazine or pyocyanin compound. However, further analyses are needed to identify the pigments.

5.3.3 Preliminary Screening of Antibacterial Activity of Pigmented Bacterial Extract

The seven pigmented bacterial strains were further screened for their antibacterial activity against five standard pathogenic bacteria using disc diffusion method, which include two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus faecalis*) and three Gram-negative bacteria (*Shigella* sp.*, Salmonella typhi* and *Escherichia coli*).

Upon incubation for 24 hours, the crude methanolic extract displayed various range of antibacterial activity against the pathogenic bacteria (Table 5.2). As experiments were carried out in triplicates (Appendix D), the zone of inhibition was expressed in standard deviation values. Among the seven bacterial isolates, four strains exhibited antagonistic activity against at least one test bacterium.

In particular, the crude methanolic extract from *Pseudomonas aeruginosa* PMCC1 and *Paracoccus zeaxanthinifaciens* PMCC2 displayed antibacterial activity against more than three pathogenic bacteria, while *Staphylococcus saprophyticus* PMDP and *Exiguobacterium profundum* PMEC2 specifically showed inhibition against *Shigella* sp.. No zone of inhibition (> 9 mm) was recorded for the extracts of another three isolates which are *Staphylococcus pasteuri* PMBS3, *Microbacterium oxydans* PMCT1 and *Acinetobacter pittii* PMDC2 against all tested bacteria.

Table 5.3. Antibacterial Activity of Crude Methanolic Extract of Pigmented Bacteria

Culture supernatant	Mean of zones of inhibition ± SD (mm)				
	Gram positive bacteria		Gram negative bacteria		
	S. aureus	***S. faecalis***	***Shigella* sp.**	***S. typhi***	***E. coli***
Positive control	11.6 ± 0.57	20.0 ± 1.73	17.6 ± 1.15	14.6 ± 0.57	19.0 ± 0.00
Negative control	-	-	-	-	-
***S. pasteuri* PMBS3**	-	-	-	-	-
***P. aeruginosa* PMCC1**	21.6 ± 0.57	22.0 ± 1.00	17.6 ± 0.57	12.6 ± 0.57	20.0 ± 0.00
***P. zeaxanthinifaciens* PMCC2**	9.33 ± 0.57	17.0 ± 1.00	13.6 ± 0.57	-	12.6 ± 0.57
***M. oxydans* PMCT1**	-	-	-	-	-
***A. pittii* PMDC2**	-	-	-	-	-
***S. saprophyticus* PMDP**	-	-	11.6 ± 0.57	-	-
***E. profundum* PMEC2**	-	-	10.0 ± 1.00	-	-

Note. (-) indicates that no inhibition zone was observed

P. aeruginosa PMCC1 showed relatively high antibacterial activity against all five tested pathogenic bacteria with the highest zone of inhibition against *S. faecalis* and *S. aureus* with 22.0 ± 1.00 mm and 21.6 ± 0.57 mm respectively, followed by *E. coli* with 20.0 ± 0.00 mm, *Shigella* sp. with 17.6 ± 0.57 mm and *S. typhi* with 12.6 ± 0.57 mm (Table 5.2). This broad antibacterial activity exhibited by *P. aeruginosa* PMCC1 agrees with previous studies regarding the inhibition of marine pigmented *P. aeruginosa* strains against selected pathogenic bacteria (Darwesh *et al.,* 2019; Darabpour *et al.,* 2010). Some strains of *Pseudomonas sp.* are known to produce pigments such as the phenazine compounds with antibiotic properties (Isnansetyo and Kamei, 2009).

The antibacterial effect of *P. aeruginosa* PMCC1 against *S. aureus* and *E. coli* is also similar with the study by Darwesh *et al.* (2019) on the inhibitory activity of pyocyanin extract from *P. aeruginosa* strain OSh1 isolated from water samples of Mediterranean Sea, Alexandria, Egypt. However, the researchers' *P. aeruginosa* OSh1 strain showed no effect against *Shigella* sp. (Darwesh *et al.,* 2019). On the other hand, El-Fouly *et al.* (2015) reported that pyocyanin purified from *P. aeruginosa* isolated from soil and clinical specimens have high antimicrobial activities against *Shigella* sp. among other pathogenic bacteria such as *S. aureus, E. coli* and *S. typhi* (El-Fouly *et al.,* 2015), similar to the result found in this study. Pyocyanin has been revealed to have a powerful inhibitory effect on bacterial growth and/or their biofilm forming ability, which can have the potential to be developed as new drugs for treatment and prevention of different bacterial infections (Feghali and Na'was, 2018).

For *P. zeaxanthinifaciens* PMCC2, methanolic extract of the yellow bacteria showed inhibition against four bacterial indicators with the zone of inhibition of 9.33 ± 0.57 mm for *S. aureus*, 17.0 ± 1.00 mm for *S. faecalis,* 13.6 ± 0.57 for *Shigella* sp. and 12.6 ± 0.57 mm for *E. coli.* Wibowo *et al*. (2019) had previously identified four bacterial strains of the *Paracoccus* genus isolated from the sea cucumbers *Stichopus japonicus* and *H. leucospilota* from Lampung, Indonesia that showed antibacterial activity against *Bacillus subtilis* and *S. aureus.* In addition, a strain of *Paracoccus marcusii* was also reported to enhance the growth performance and immunity of juvenile sea cucumber *Aspotichopus japonicus*, hence are being developed as potential probiotics for the sea cucumbers (Yan *et al*., 2014; Yang *et al*., 2015). However, these other *Paracoccus sp.* have not been reported to be pigmented, and not much studies have been carried out on antibacterial activity of yellow-producing *P. zeaxanthinifaciens.*

Some strains of *P. zeaxanthinifaciens* were previously revealed to produce zeaxanthin, a yellow-coloured carotenoid pigment. Zeaxanthin is known for its strong antioxidant and anti-cancer properties, hence is also used in the food, pharmaceutical and nutraceutical industries (Zhang *et al.,* 2018). Since the crude extract from *P. zeaxanthinifaciens* PMCC2 showed inhibition against Gram positive and Gram-negative pathogenic bacteria (Table 5.2), therefore further determination of its antimicrobial activity could also be beneficial for its potential use in the biotechnological industries.

Another two pigmented bacterial isolates namely the yellow-coloured *S. saprophyticus* PMDP and orange bacterial strain *E. profundum* PMEC2 showed inhibition only against *Shigella* sp. with 11.6 ± 0.57 mm and 10.0 ± 1.00 mm

respectively. Even though there are yet no research found regarding antibacterial activity of pigmented *S. saprophyticus,* previous studies have documented on the antimicrobial activity of non-pigmented marine *S. saprophyticus* isolates. A recent study by Bibi *et al.* (2020) reported that a *S. saprophyticus* strain isolated from marine sponges in the Red sea in Jeddah, Saudi Arabia did not show inhibition on *S. aureus, E. coli* and *E. faecalis,* but displayed antagonistic activity against fungi *Phytophthora capsici* and *Pythium ultimum.* However, another study by Mani *et al*. (2016) showed that a novel biosurfactant-producing *S. saprophyticus* strain isolated from coastal sites of Puducherry, India exhibited excellent antibacterial and antifungal activities, such as inhibition against *Klebsiella pneumoniae*, *E. coli* and *Vibrio cholerae.*

In addition, the yellow staphyloxanthin pigment produced by *Staphylococcus sp.* was previously elucidated to have antimicrobial and strong antioxidant activities (Liu *et al.,* 2005). For example, purified staphyloxanthin from a *S. aureus* strain displayed antibacterial activity against *Shigella dysenteri* among other bacterial species including *Staphylococcus epidermidis, Acinetobacter baumanii* and *Proteus mirabilis* (Alshamaa & Issam, 2017). Besides, staphyloxanthin pigment from *Staphylococcus gallinarum* was also shown to have *in vitro* anticancer activity against 4 different cancer cell lines and less cytotoxicity towards normal non-cancerous cell lines, suggesting its potential to be a therapeutic agent (Barretto & Vootla, 2018), providing its safety has been assessed.

The antibacterial activity of *E. profundum* PMEC2 isolated from *H. hilla* cuticle against *Shigella* sp. in this study is in line with a previous study by Balraj *et al.* (2014) for the activity of orange pigment purified from a marine *Exiguobacterium* sp. isolated

from the Peninsular Region of India. However, the authors' strain also showed antimicrobial activity against other bacteria including *E. coli* and *S. aureus*, contrasting with this study. This could be due to the different pigments produced by the two strains, consistent with the observation of the different maximum absorption peak when analysed using UV-visible spectrophotometer (Section 5.3.2). Another research by Alipiah *et al*. (2016) have also isolated a strain of *E. profundum* from the surface of sea cucumber *Stichopus badionotus* from the west coast of peninsular Malaysia, which did not exhibit antagonistic effect against four pathogenic *Vibrio* sp. However, the *E. profundum* strain was not reported to be pigmented.

From the antibacterial activity screening in this study, the Gram-negative *Shigella* sp. was observed to be the most susceptible test bacteria to crude pigment extracts since it is affected by *P. aeruginosa* PMCC1, *P. zeaxanthinifaciens* PMCC2 *S. saprophyticus* PMDP and *E. profundum* PMEC2. Moreover, the Gram-positive *S. aureus* and *S. faecalis* were highly affected by pigment extract from *P. aeruginosa* PMCC1. This variation in the sensitivity or susceptibility towards antimicrobial compounds could be due to the differences in lipid content of cell wall of Gram-positive and Gram-negative bacteria (El-Fouly *et al.*, 2015). However, certain other factors can also affect these antimicrobial results by disc diffusion technique including the inoculum, media composition and depth, delay between application of the disc and incubation, temperature, atmosphere and duration of incubation, generation time, the antibiotic concentration of the disc and the method of reading zone (Hedges, 1999; Gould, 2000). Therefore, further evaluations are needed for verification of antimicrobial activity of these pigment extracts against pathogenic bacteria.

5.4 Conclusion

Seven pigmented bacterial strains isolated from sea cucumbers and marine sediment were evaluated in terms of their colony colours observation, spectrophotometric analysis of the crude pigment extracts and screened for their antibacterial activity. Extracts from two of the yellow bacterial strains which are *S. saprophyticus* PMDP and *M. oxydans* PMCT1, and the pink *A. pittii* PMDS1 showed absorbance peaks at the region of 400 to 500 nm on UV-visible spectrophotometry, which are in the range of visible spectra for carotenoid pigments, while the green extract from *P. aeruginosa* PMCC1 exhibited absorbance spectrum resembling pyocyanin or other phenazine-based pigments. However, further analyses are required to confirm these pigments such as using HPLC and GCMS, as well as to determine the orange pigment extract from *E. profundum* PMEC2 and optimize yellow pigment production and extraction by the other two bacterial strains, *P. zeaxanthinifaciens* PMCC2 and *S. pasteuri* PMBS3. Preliminary analysis of the antibacterial activity of crude pigment extract suggested that *P. aeruginosa* PMCC1 and *P. zeaxanthinifaciens* PMCC2 have efficient antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria, while *S. saprophyticus* PMDP and *E. profundum* PMEC2 have antibacterial activity against *Shigella* sp.. Further evaluation is needed to verify the antimicrobial activity by evaluating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these pigment extracts. These pigments can also be further purified and characterized for their potential use in industries.