Jurnal Kejuruteraan dan Sains Kesihatan
Journal of Engineering and Health Sciences
Jilid 4 2020: 111 - 122

ISOLATION OF BACTERIA ASSOCIATED WITH THE TENTACLES OF *HOLOTHURIA (LESSONOTHURIA) PARDALIS* (SELENKA 1867) FROM PULAU TINGGI, JOHOR INCLUDING PIGMENT-PRODUCING *CHRYSEOBACTERIUM* SP.

Siti Najihah Solehin[a*], Nor Shahida Ab Rahman[a], Muhammad Abdul Latiff Abu Bakar[a], 'Aisyah Mohamed Rehan[b], Kamarul Rahim Kamarudin[a]

[a]*Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology (FAST), Universiti Tun Hussein Onn (UTHM)*
[b]*Department of Chemical Engineering Technology, Faculty of Engineering Technology (FTK), Universiti Tun Hussein Onn (UTHM)*

Abstract

Pigment-producing microorganisms have become a main focus due to the search of food-grade microbial pigments for natural food colourant industry. Therefore, this study aimed to isolate and genetically identify bacteria including pigment-producing bacterial strains associated with the tentacles of *Holothuria (Lessonothuria) pardalis* (Selenka 1867) from Pulau Tinggi, Johor. Morphological characteristics of bacteria based on Gram staining were observed using a digital compound microscope. Partial 16S ribosomal RNA (rRNA) gene sequencing was also incorporated to support the morphological approach. A number of six partial gene sequences of non-protein coding 16S rRNA were obtained and the Basic Local Alignment Search Tool (BLAST) analysis suggested the presence of three known bacteria species from the genera of *Pseudomonas, Chryseobacterium* and *Klebsiella*. The neighbourhood-joining method and Maximum Likelihood method used in the phylogenetic analysis supported the identification and classification of the bacteria species. Among the bacterial isolates, only strain B14 identified as *Chryseobacterium sp*. produced pigment i.e. orange pigment. The highest point of cell density of *Chryseobacterium sp*. was recorded at 16 hours. However, further analysis is needed to confirm the pigment characterisation and the potential of this pigment to be exploited as natural colourant in the food industry.

Keywords: *Chryseobacterium* sp., *Holothuria (Lessonothuria) pardalis*, Gram staining, 16S ribosomal RNA gene, phylogenetic analysis, microbial pigment

1.0 INTRODUCTION

Sea cucumber is an invertebrate organism with leathery skin and elongated body, and they can be found in intertidal sediment (Majid *et al.*, 2012). It is one of the deposit-feeders as it filtrates sediment for organic particles, since a lot of microalgae, seaweed, and benthic organisms were identified in its gut content (Anissuzaman *et al.*, 2018). More than 170 researches on Malaysian sea cucumbers (Phylum Echinodermata: Class Holothuroidea) have been recorded to date. Sea cucumber has been used as a food source and traditional medicine (Borbar *et al.*, 2011). Beside that, Malaysia has been ranked fourth top producer after Indonesia, China and the Philippines in sea cucumber commercialisation (Kamarudin *et al.*, 2017).

Sea cucumbers can produce biologically active secondary metabolites resulted from their live adaptation in extreme environments (Pangestuti & Arifin, 2018). In 2013, 37 bacterial species were identified from the respiratory tree of *Holothuria (Mertensiothuria) leucospilota* that was collected from Pulau Pangkor, Malaysia (Kamarudin *et al*., 2013). Furthermore, in 2015, there were 14 species of bacteria found in the coelomic fluid of *H. leucospilota and Stichopus chloronotus* collected from Pulau Dayang Bunting and Pulau Tioman (Lukman *et al*., 2015). Meanwhile, in 2018, 24 species of bacteria were found in the gastrointestines of *H. leucospilota and Stichopus horrens* collected from Pulau Pangkor (Kamarul *et al*., 2018). Thus, the association and interaction between microorganisms and sea cucumber have recently become a great attention.

In this study, *Holothuria (Lessonothuria) pardalis* was selected considering its high distribution in the sampling location in Pulau Tinggi, Johor. The presence of bacterial isolates associated with the tentacles of *H. pardalis* were recorded in this study and the growth curve of a pigment-producing bacterium isolated from the tentacle part was documented. In fact, pigment-producing bacteria isolated from sea cucumber were rarely recorded. Kamarudin *et al.* (2013) isolated a pigment-producing bacterium *Staphylococcus kloosii* from the respiratory tree of *H. leucospilota*. Nonetheless, further analysis is needed for the pigment-producing bacterium isolated in this study in order to confirm its pigment characterisation and the potential to be exploited as natural colourant in the food industry.

2.0 MATERIALS AND METHOD

2.1 Sampling Site

Holothuria (Lessonothuria) pardalis (Selenka 1867) specimens were collected from Pulau Tinggi, Johor, Malaysia. The sampling was done during low tide and no fixed or standard sampling hours were allocated (Figure 1). Specimens were kept in an icebox containing seawater before transferred to the laboratory for bacterial isolation.

Figure 1: Sampling location of *Holothuria (Lessonothuria) pardalis* in Pulau Tinggi, Johor. Source: Google Earth.

2.2 Isolation of Bacteria

H. pardalis specimen was dissected with a sterile blade in Biological Safety Cabinet (BSC) to avoid possible contamination from surrounding. All bacteria strains were isolated from four external body parts of the specimen, i.e. cuticle, ventral podium, anus and tentacle; and also from the coelomic fluid of *H. pardalis* and bottom sediment. Each of the specimen body parts and the other bacterial sources was swabbed using a cotton swab and spread onto Tryptone Glucose Yeast Extract (TGYE) agar (casein enzymatic hydrolysate, 5g/l; yeast extract, 3g/l; glucose, 1 g/l; agar, 15g/l at pH 6.8). After overnight incubation at 30^{o}C (as seawater temperature recorded in Pulau Tinggi was 30^{o}C at average), the morphologies of the bacterial colonies were observed and different colonies were repeatedly sub-cultured in new TGYE agar in order to purify each single target bacterium. Each pure colony was observed for their characteristics like shape, colour and texture based on the Gram staining method. Microscopic observation and documentation were done using Olympus System Microscope Model BX

43W with the magnification of 1000x (with 10x eyepiece and 100x objective lens).

2.3 DNA Extraction, PCR Amplification and Phylogenetic Analysis

Microbial DNA was directly extracted using the boiling method at 98^0C for 15 min. Two universal primers, PB36 (forward) and PB38 (reverse) were used for partial 16S rRNA gene amplification (Bell *et al*., 1999)

PB36 (forward) – 5' – AGRGTTTGATCMTGGCTCAG – 3' (20 bases)
PB38 (reverse) – 5' – GKTACCTTGTTACGACTT – 3' (18 bases)

Standard polymerase chain reaction (PCR) was done by using 25µl reaction volume containing ExTEN 2x PCR master mix 12.5 µl, each primer 0.5 µl, DNA template 2.5 µl and ultrapure water 9 µl. PCR amplification was performed for 35 cycles, with the following conditions: An initial denaturation at 95°C for 4 min, 95°C for 30 s (DNA denaturation), 59°C for 30 s (the optimized temperature for annealing) and 72°C for 45 s (DNA extension), with a final extension at 72°C for 10 min and the temperature was then held at 4ºC until the samples were taken out. Unpurified PCR products were sent to Apical Scientific Sdn Bhd, Serdang for PCR products purification and partial DNA sequencing. MEGA-X 10.1 software was used to analyse the DNA sequences. The partial 16S rRNA gene sequences obtained were matched with the corresponding sequence from the GenBank Database using the Basic Local Alignment Search Tool (BLAST) available at the National Centre for Biotechnology Information (NCBI), US National Library of Medicine. The reconstruction of the Neighborhood-Joining (NJ) phylogenetic tree and Maximum Likelihood (ML) phylogenetic tree were done for further bacteria species identification and classification.

2.4 Bacterial Growth Measurement

Pigment-producing bacterium was selected by naked eyes based on the colony colour on the growth medium. The selection was also done based on previous studies especially on the exclusion of pathogenic strains. After the selection, an inoculum starter was prepared with a pure strain of pigment-producing bacterium was grown in 50 ml of TGYE broth medium (casein enzymic hydrolysate, 10g/l; glucose, 5g/l; yeast extract,1g/l; dipotassium phosphate, 1.25 g/l; pH 6.8) at 30ºC and 150 rpm for 24 hours. Furthermore, 5 ml of the inoculum starter was added into 45 ml of TGYE broth and then incubated at 30ºC and 150 rpm for 48 hours.

By using UV-Vis spectrophotometer, the optical density of 1 ml of the TGYE broth containing the pigment-producing bacterial cells was measured at 600nm for every hour in order to observe the bacterial cell growth.

3.0 RESULTS AND DISCUSSION

A number of 26 pure bacterial strains were observed and isolated from the source samples. Table 1 shows the morphological data observed using the Olympus System Microscope Model BX 43W. Among the bacterial isolates, only strain B14 identified as *Chryseobacterium* sp. was observed with pigment production i.e. orange pigment (Fig. 2).

Table 1: Morphological data observed using the Olympus System Microscope Model BX 43W

Source of Bacteria	Microbial Isolates	Number of Bacterial Colonies	Bacterial Shape	Gram Positive	Negative
Bottom sediment	A11	Few	Rods		√
Tentacles	B12	Many	Streptococcus	√	
	B11	Many	Staphylococcus	√	
	B13	Moderate	Rods		√
	B14	Moderate	Rods		√
Anus	C11	Few	Diplococci	√	
	C12	Few	Cocci	√	
	C13	Many	Staphylococcus	√	
Coelomic fluid	D11	Moderate	Streptococcus	√	
Intestine	E11	Many	Rods		√
	E12	Moderate	Diplococci	√	
Respiratory tree	F11	Few	Cocci		√
	F13	Moderate	Streptocioccus		√
Cloaca	G112	Many	Staphylococcus	√	
	C1	Few	Rods		√
	C4	Moderate	Staphylococcus		√
External part	H12	Many	Staphylococcus	√	
	H11	Many	Staphylococcus	√	
	H12	Many	Staphylococcus	√	
Stomach	J11	Moderate	Streptococcus		√
	S1	Many	Staphylococcus	√	

	S2	Many	Streptobacillus	√
	S3	Many	Streptobacillus	√
Podium	K11	Few	Diplococcus	√
	K12	Moderate	Streptococcus	√
	M2	Many	Staphylococcus	√

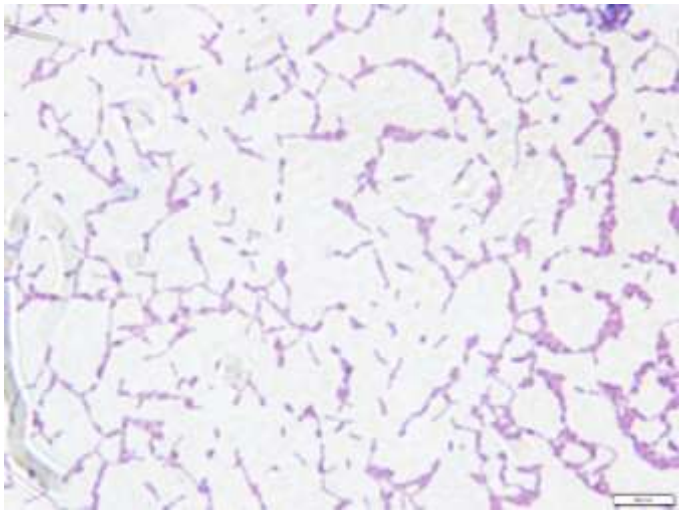

Figure 2: Gram-stained B14 strain under Olympus System Microscope Model BX 43W with magnification of 1000x. The pink colour from Gram staining indicates B14 strain as a Gram-negative bacterium with rod shape.

Among the bacterial isolates, only six isolates were shortlisted for DNA sequencing based on the PCR results. Thirty corresponding sequences from the GenBank, NCBI were incorporated for the phylogenetic analyses. Based on the NJ tree and ML tree shown in Figures 3-4, the bacterial isolates (A11, B12, B13, B14, E13, and H12) could be identified up to genus and species level. Bootstrapping (Felsenstein, 1985) with 1000 replicate data set was used to estimate the phylogenetic confidence. NJ tree and ML tree indicated that A11 and B13 were clustered together in genus *Pseudomonas* with a bootstrap values of 98% (NJ) and 67% (ML) respectively. Apart from that, E13 was closer to the genus *Klebsiella* with robust bootstrap values of 100% as shown by both phylogenetic trees (Figures 3-4). Likewise, B14 was clustered in genus *Chryseobacterium* with robust bootstrap values of 100% for both NJ tree and ML tree; and the closest species was *Chryseobacterium gleum*. H12 and B12

were grouped in clusters other than the genera of *Cryseobacterium*, *Pseudomonas* and *Klebsiella.*

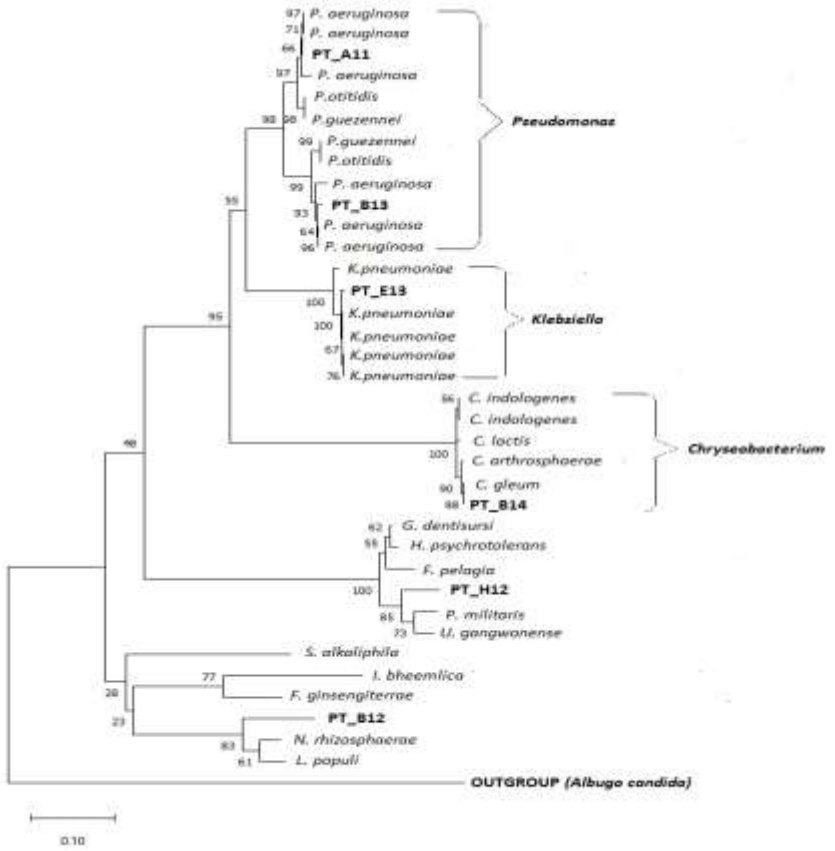

Figure 3: Neighbour-joining (NJ) tree of six bacteria associated with *H. pardalis* from Pulau Tinggi, Johor as inferred from partial 16S rRNA mtDNA gene sequences using MEGA-X version 10.1. A number of 30 sequences from the GenBank, NCBI, U.S. National Library of Medicine were also incorporated. *Albugo candida* was used as an outgroup to define the root of tree. Bootstrap values > 50% with 1000 replicates are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

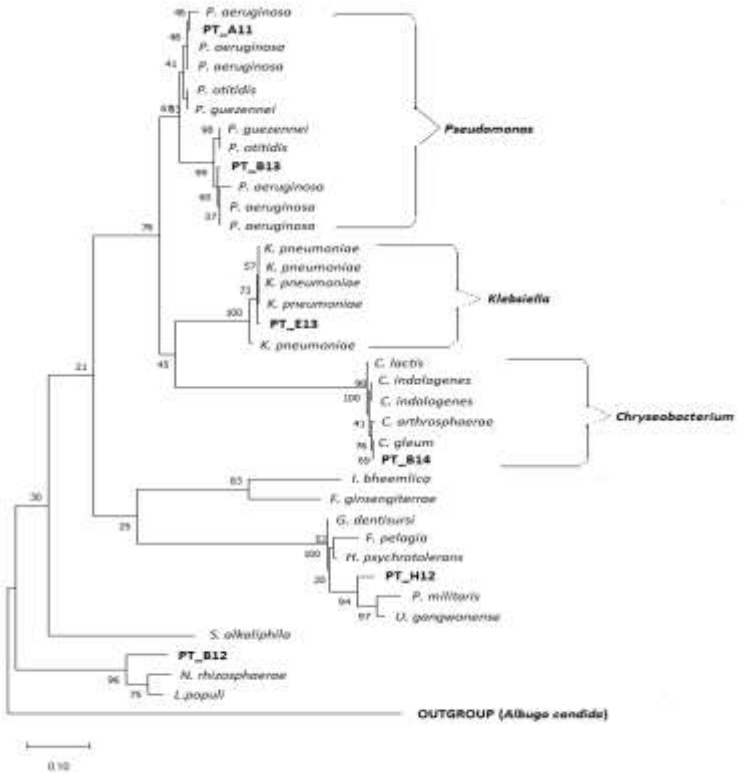

Figure 4: Maximum Likelihood (ML) tree of six bacteria associated with *H. pardalis* from Pulau Tinggi, Johor as inferred from partial 16S rRNA mtDNA gene sequences using MEGA-X version 10.1. A number of 30 sequences from the GenBank, NCBI, U.S. National Library of Medicine were also incorporated. *Albugo candida* was used as an outgroup to define the root of tree. Bootstrap values > 50% with 1000 replicates are shown next to the branches. The scale bar represents nucleotide substitutions per site.

3.1 Cell Growth

Among the six bacterial isolates, only B14 was observed with pigment production; therefore it was selected for cell growth analysis. B14 was phylogenetically identified as *Chryseobacterium* sp.. In fact, genus *Chryseobacterium* was firstly introduced by Vandamme *et al.* (1994) and it was regarded previously as *Flavobacterium* member. Members of the genus *Chryseobacterium* are typically Gram staining negative, non-motile, rods shaped and orange-red compound (Nguyen *et al.,* 2013). Figure 5 shows that the

exponential phase of the cell growth occurred until 16 hours of incubation, by which at that stage it showed the highest or maximum point of cell density. According to Kamarudin *et al.* (2013), pigment production started during the exponential cell growth phase. After 16 hours of incubation, the cell density level started to decline. The cell growth curve is important for scaling up microbial pigment production especially for industrial usage.

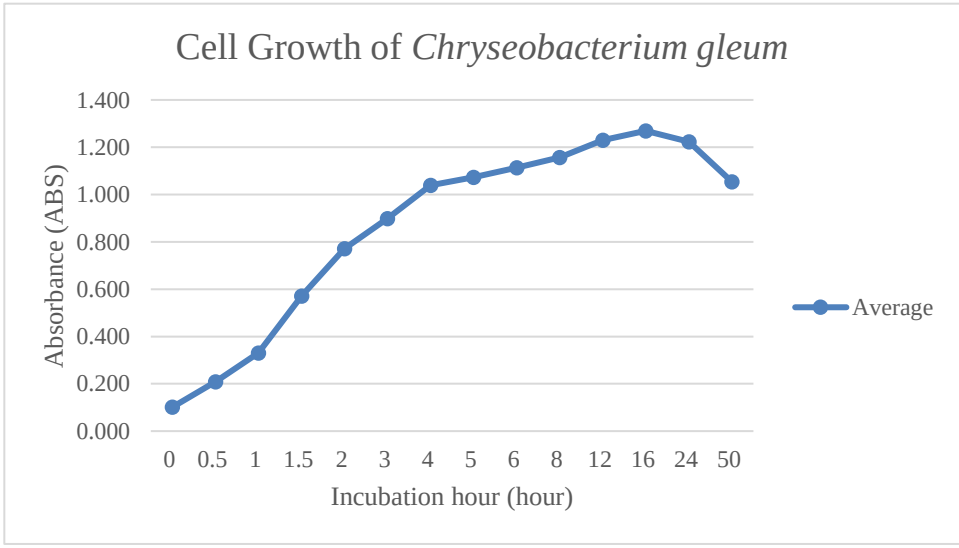

Figure 5: Cell growth curve of pigment-producing B14 strain for 48 hours.

4.0 CONCLUSION

A number of 26 pure colonies of bacteria associated with *H. pardalis* from Pulau Tinggi, Johor were documented, but only six strains were managed to be sequenced and included in the phylogenetic tree reconstruction. This study also recorded the presence of one pigment-producing bacterium i.e. B14 strain identified as *Chryseobacterium* sp.. Besides, the highest point of cell density of the pigment-producing bacterium was recorded at 16 hours. Further analysis should be conducted to extract and characterise the orange pigment of B14 strain and the potential of this pigment to be exploited as natural colourant in the food industry.

ACKNOWLEDGEMENT

This study was funded by the Industrial Grant of Universiti Tun Hussein Onn Malaysia (UTHM) with code no. M004, Fundamental Research Grant Scheme

(FRGS) from the Malaysian Ministry of Education (FRGS/1/2019/WAB09/UTHM/03/2) and partially supported by Postgraduate Research Grant (GPPS) UTHM with code no. H416. It is part of a thesis which was to be submitted as partial fulfilment to meet requirements for the Doctor of philosophy in Science at UTHM.

REFERENCES

Anisuzzaman, M. D., Jeong, U. C., Jin, F., Kabery, K., Choi, J. K., Lee, D. I, Yu, H.S., & Kang, S. J. (2018). Effects of *Ulva lactuca* and *Laminaria japonica* Algae in Prepared Feeds on Growth, Survival, Fatty Acid Compositions and Interleukin (IL)-10 Production of Sea Cucumber *Apostichopus japonicus*. *Int J Fish & Aqua Stu*, 6(2), 387-395.

Bell, P. J. L., Nevalainen, H., Morgan, H. W. & Bergquist, P. L. (1999).Rapid Cloning of Thermoalkalaophilic Lipases from *Bacillus spp*. *Biotechnol Lett* (21), 1003–1006

Bordbar, S., Anwar, F., & Saari, N. (2011). High-Value Components and Bioactives from Sea Cucumbers for Functional Foods—A Review. *Marine drugs*, 9(10), 1761-1805.

Dufossé, L. (2016). Current and Potential Natural Pigments from Microorganisms (Bacteria, Yeasts, Fungi, Microalgae). In *Handbook on Natural Pigments in Food and Beverages* (pp. 337-354). Woodhead Publishing.

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, 39(4), 783-791.

Kamarudin, K. R., Rehan, M. M., & Bahaman, N. A. (2017). Morphological and Molecular Identification of Sea Cucumber Species *Holothuria scabra*, *Stichopus horrens* and *Stichopus ocellatus* from Kudat, Sabah, Malaysia. *Pertanika Journal of Tropical Agricultural Science*, 40(1), 161-172.

Kamarudin, K.R., Ngah, N., Tengku Abdul Hamid, T.H., & Susanti, D. 2013. Isolation Of A Pigment-producing Strain of *Staphylococcus Kloosii* from the Respiratory Tree *of Holothuria (Mertensiothuria) leucospilota* from Malaysian Waters. *Tropical Life Sciences Research*, 24(1), 85-100.

Kumar, A., Vishwakarma, H. S., Singh, J., Dwivedi, S., & Kumar, M. (2015). Microbial Pigments: Production and Their Applications In Various

Industries. *International Journal of Pharmaceutical, Chemical and Biological Sciences*, 5(1), 203-212.

Lukman, A. L., Nordin, N. F. H., & Kamarudin, K. R. (2014). Microbial Population in the Coelomic Fluid of *Stichopus chloronotus* and *Holothuria (Mertensiothuria) leucospilota* Collected from Malaysian waters. *Sains Malaysiana*, 43(7), 1013-1021.

Majid, A., Maryam, E., Reza, D. A., Neda, S., & Ghodrat, M. (2012). New Observation of Two Sea Cucumber Species from Abu Musa Island (Persian Gulf, Iran). *Eur J Exp Biol*, *2*(3), 611-615.

Nguyen, N. L., Kim, Y. J., Hoang, V. A., & Yang, D. C. (2013). *Chryseobacteriumginsengisoli sp. nov*., isolated from the rhizosphere of ginseng and emended description of *Chryseobacterium gleum. International journal of systematic and evolutionary microbiology*, 63(8), 2975-2980.

Pangestuti, R., & Arifin, Z. (2018). Medicinal and Health Benefit Effects of Functional Sea Cucumbers. *Journal of traditional and complementary medicine*, 8(3), 341-351.

Tamura K., Nei M., and Kumar S. (2004). Prospects for Inferring Very Large Phylogenies by Using the Neighbor-Joining Method. *Proceedings of the National Academy of Sciences (USA)* 101:11030-11035.

Vandamme, P., Bernardet, J. F., Segers, P., Kersters, K., & Holmes, B. (1994). New Perspectives in the Classification of the Flavobacteria: Description of *Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev*. *International Journal of Systematic and Evolutionary Microbiology*, 44(4), 827-831.

Vila, E., Hornero-Méndez, D., Azziz, G., Lareo, C., & Saravia, V. (2019). Carotenoids from Heterotrophic Bacteria Isolated from Fildes Peninsula, King George Island, Antarctica. *Biotechnology Reports*, 21, e00306.