RESEARCH ARTICLE

Antibacterial Activity of Carotenoid Pigments Produced by Heterotrophic Bacteria from Seawater in Krakal Coastal Area, Yogyakarta, Indonesia

Rina Septriani Sidin[1] and Endah Retnaningrum[2,3*]

OPEN ACCESS

[1] Graduate Student, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan Sekip Utara, Sleman, Yogyakarta, 55281, Indonesia

[2] Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Sleman, Yogyakarta, 55281, Indonesia

[3] Indonesia Natural Dye Institute (INDI), LPPT, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia

*Corresponding Author:
endahr@ugm.ac.id

Received: 21 May 2022

Accepted: 25 July 2022

Published: 30 August 2022

Academic Editor: Dr. Dewi Seswita Zilda

 Accreditation Number:148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. https://doi.org/ 10.15578/squalen.648

Abstract

Investigating natural pigments resulting from marine bacterial secondary metabolites is important because of their broad benefits in the food, pharmaceutical, cosmetic, and textile industries. In this study, 52 isolates of pigmented bacteria were isolated from seawater in the Krakal coastal area, Yogyakarta, Indonesia. Seven bacterial isolates showed high pigment stability. Profiling of pigment extracts using a mixture of acetone and methanol with UV–Vis spectrophotometric reading showed that the compounds produced were carotenoids. The highest total carotenoid content with UV–Vis spectrophotometric reading at 480 nm was produced by the Kral-3 isolate (1800 µg/g), followed by Kral-15 (1698.9 µg/g) and Kral-25 (797.2 µg/g) isolates. Analysis of the antibacterial activity of the carotenoid extracts of Kral-3, Kral-15, and Kral-25 isolates using the agar well diffusion method revealed a very strong inhibition score against *Escherichia coli*. In addition, the carotenoid extract of the Kral-3 isolate showed a strong inhibition score against *Staphylococcus aureus*, whereas the Kral-15 and Kral-25 isolates showed moderate inhibition scores. Based on the 16S rRNA gene analysis, the Kral-3, Kral-15, and Kral-25 isolates were identified as *Kocuria rhizophilla*, *Calidifontibacter* sp., and *Rhodococcus ruber*, respectively. Based on the findings, the novel bacterial strains are a potential bioresource for the commercial production of natural carotenoids.

Keywords: secondary metabolites, pigment extracts, inhibition score, *E. coli*, *S. aureus*

Introduction

Natural pigments are compounds with specific molecular structures of a chromophore, which play an important role in the physiological and molecular processes of microorganisms, including sun radiation protection and adaptation to extreme environments. Given the unique nature of these microorganism pigments, they have been widely used in various fields, such as the food, pharmaceutical, cosmetic, and textile industries (Usman et al., 2017). In addition, natural pigments produced by microorganisms, particularly bacteria, are safer, biodegradable, and easily extracted from cell biomass, and they have high pigment stability. Moreover, they can be produced quickly, easily, and cheaply through microbial cultivation, and their genes can easily be modified related to the pigment synthesis pathway (Narsing et al., 2017; Velmurugan et al., 2020).

Carotenoid are bacterial pigments which are sought by consumers and are needed to date. Carotenoids can improve human health because of their role in many metabolic functions of the body and antibacterial, antioxidant, anti-inflammatory, and anticancer properties (Ambati et al., 2014; Kirti et al., 2014; Esatbeyoglu & Rimbach, 2017). Carotenoids are composed of a symmetric C40 tetraterpenoid hydrocarbon compound consisting of eight isoprene units (polyisoprene) which have a C5 isoprene member that produces C40 carotenoids (Aryee et al., 2018). This structure produces colors such as yellow, orange, and red (Kirti et al., 2014).

Several researchers have obtained carotenoids from bacteria with antibacterial abilities. Mohana et al. (2013) reported that red and yellow pigments extracted from bacteria species of *Micrococcus luteus* and *M. roseus* could inhibit the growth of *S. aureus* and *S. faecalis*, respectively. Meanwhile, Ravikumar et al. (2016) reported that the carotenoids obtained from *Holomonas* sp. showed antibacterial activity against five pathogenic bacteria, including *Klebsiella* sp., *S. aureus*, *Pseudomonas aeruginosa*, *S. pyogens*, and *E. coli*. Moreover, *Kocuria roseus*, *Bacillus* sp., *Staphylococcus* sp., *Corynebacterium* sp., and *Brevibacterium* sp. have been analyzed and proven to produce carotenoids and inhibit pathogenic bacteria *S. aureus* ATCC 25923, *S. xylosus*, *Peanibacillus macerans*, and *Citrobacter divesus* (Boontosaeng et al., 2016). Sibero et al. (2019) have isolated *Vibrio oweensii* TNKJ.CR.24-7 from coral samples of Karimunjawa National Park, Jepara Region, Central Java Province, Indonesia. *V. oweensii* TNKJ.CR.24-7 produced ß-carotene and inhibited several pathogenic bacteria, including ESBL *E. coli*, *Klebsiella pneumoniae*, and methicillin-resistant *S. aureus*.

Organic and recalcitrant materials in coastal environments are high because of human activities. Consequently, the abundance and diversity of heterotrophic bacteria in the environment are also high. Therefore, bacteria play a key role in recycling these materials through the "microbial loop" (Liang et al., 2021). In addition, exposure to ultraviolet light from sunlight and salinity causes pigmented heterotrophic bacteria to be more adaptable and dominant in such environments.

Carotenoid pigments produced by these heterotrophic bacteria play a role in cell adaptation, protecting cells from UV radiation and oxidative damage. These carotenoids are involved in the membrane fluidity mechanism to regulate cell nutrient transport (Rodríguez-Concepción et al., 2018). Since carotenoids are secondary metabolites, bacteria will produce these compounds in the stationary phase. They are not associated with cell growth and replication (Thanapimmetha et al., 2017; Srinivas et al., 2021). Research on the antibacterial activity of bacterial carotenoids on heterotrophic bacteria from seawater in the Krakal coastal area, Yogyakarta, Indonesia, has not been reported. Therefore, this study aimed to obtain heterotrophic bacteria that produce carotenoids from seawater in the Krakal coastal area, Yogyakarta, Indonesia. The total concentration and antibacterial activity of carotenoid extract were determined to confirm the potential application of bacterial isolates in human health.

Materials and Methods

Sample Collection and Isolation of Pigmented Heterotrophic Bacteria

Seawater samples were collected in 25 mL sterile conical tubes from the Krakal coastal area, Yogyakarta, Indonesia (8°082 46.23 S 110°352 53.53 E), in September 2021. A total of 0.1 mL of sample, serially diluted with buffered saline, was inoculated on 15 mL Zobell Marine Agar 2216 media (Himedia) using the spread plate method. After incubation for 72 h at 30 °C, pigmented bacterial colonies (yellow, orange, pink, or red) were selected and purified periodically, and the stability of the pigment color was observed (Asker, 2018). Then, bacterial isolates were characterized based on the colony and cell morphology.

Biomass, Pigment Extraction, and Carotenoid Determination

The pigmented bacterial isolates were cultured in a Zobell Marine Broth 2216 medium with shaking at 150 rpm and 30 °C for 144 h. The bacterial cells were collected by centrifuging 5 mL of culture broth at 5000 g for 20 min. The cell pellets were washed with distilled water to remove salts, centrifuged, and then dried at 60 °C using an oven until a constant weight was reached. The total biomass was determined as grams of dry cells per liter of media. In addition, dry cells were suspended in sterile saline and lysed by adding lysozyme (20 mg/mL). Carotenoids were extracted using acetone and methanol in a 7:2 ratio and incubated on a rotary shaker (100 rpm) at room temperature for 24 h in the dark until the cells were bleached (Asker et al., 2018). Extracted samples were centrifuged at 5000 *xg* for 10 min, and the obtained supernatant was identified as carotenoids based on UV-Vis spectra characteristics at λ 200–900 nm.

Meanwhile, the concentration of total carotenoids was determined by reading the absorbance of solvent using UV-Vis spectrophotometry at λ 480 nm. The pellet cells were then dried to constant weight in an oven at 60 °C to measure their biomass. Furthermore, the total carotenoid concentration is determined using the following equation (Zhao et al., 2019):

$$TCC = \frac{A \times D \times V}{E \times W}$$

Where TCC is the total carotenoid content (μg/g biomass); A is the absorbance of the total carotenoid extract at 480 nm; D is the sample dilution ratio; V is the volume of the extraction solvent (mL); E is the extinction coefficient of total carotenoid (0.16), and W is the biomass dry weight of bacterial cells (g).

Carotenoid Antibacterial Activity Test Using Agar Well Diffusion

In measuring the antibacterial activity, 0.5 mg of the extracted carotenoid samples were prepared by diluting them with 3 mL of dimethylformamide (DMF). One hundred milliliters of pathogenic bacterial culture of *E. coli* FNCC 0049 and *S. aureus* FNCC 0047 (https://cfns.ugm.ac.id/wp-content/uploads/sites/861/2020/11/Catalog-FNCC-2020-fix.pdf) with a cell number of 10^8 CFU/mL was inoculated by using the pour plate method on the medium. After the media solidified, wells were made using a sterile coring drill. A total of 40 µL of pigment extract, positive control, and negative control were introduced in each well. Chloramphenicol (10 µg/mL) and DMF were used as positive and negative controls, respectively. Antibacterial activity was observed based on an inhibition zone around the well after incubation at 37 °C for 24 h (Sinha et al., 2017). The inhibitory effect of carotenoid extract was classified as low if $1 < IS < 3$, moderate if $3 \leq IS < 5$, strong if $5 \leq IS < 7$, and very strong if $7 \leq IS < 9$. The inhibition score (IS) was calculated using the following formula (Tremonte et al., 2017).

$$IS = \frac{\text{Inhibition zone diameters (mm)}}{\text{well diameter (mm)}}$$

Characterization of Bacterial Isolates Based on the 16S rRNA Gene

The pigment bacteria that produced the highest total carotenoid extract and the extract that showed the highest antibacterial activity were characterized molecularly through three stages: DNA isolation and purification, DNA amplification based on the 16S rRNA gene, and DNA sequencing and phylogenetic tree reconstruction. In this study, isolated DNA was extracted using the Quick DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was carried out using universal primers 27F (5'- AGA GTTTGATCMTGGCTCAG-3') and 1492R (5'-TAC GGYTACCTTGTTACGACTT-3'). The PCR T100™ Thermal Cycler was conditioned at 95 °C for 30 min (pre-denaturation), followed by 30 cycles consisting of 95 °C for 30 s for denaturation, 57 °C for 1 min of annealing, 72 °C for 1 min of extension, and final extension for 10 min at 72 °C. The PCR product was analyzed using 1% agarose-gel-electrophoresis and visualized using a UV transilluminator. The PCR products were then purified and sequenced using the Sanger Method (Sanger et al., 1977). Afterward, the sequencing results were validated using the Sequence Alignment Editor Bioedit program to obtain contig sequences. The 16S rRNA gene was then compared with the database in the *Basic Local Alignment Search Tool* (BLAST) program (www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree construction was analyzed using the neighbor-joining method with MEGA X and 1000 bootstrap values (Kumar et al., 2018).

Statistical Analysis

Data were obtained by triplicate measurements and determined statistically by one-way analysis of variance followed by the Duncan Multiple Range Test at $p < 0.05$.

Results and Discussion

Colony and Cell Morphological Characteristics of Pigmented Heterotrophic Bacteria

Fifty-two pigmented heterotrophic bacteria were isolated from seawater in the Krakal coastal area, Yogyakarta, Indonesia. Seven isolates with the best color stability during repeated subcultures were selected of all the bacterial isolates obtained. The colonies' colors of the seven bacterial isolates, namely, Kral-25, Kral-3, Kral-15, Kral-4, Kral-18, Kral-16, and Kral-29, were orange, bright yellow, dark yellow, pale yellow, orange-yellow, brown yellow, and beige, respectively (Figure 1). The bacterial isolates showed a diversity of colony and cell morphology (Table 1). Five were gram-positive bacteria and two were gram-negative bacteria (Sidin & Retnaningrum, 2022).

Biomass, Carotenoid Production, Total Concentration, and Carotenoid Profile from Heterotrophic Bacteria

Figure 2 shows the measurement results of biomass, carotenoid production, and total carotenoid concentration of heterotrophic bacterial isolates after 144 h of incubation. The results showed the same trend: bacterial cell biomass, pigment production, and total carotenoid concentration of seven bacterial isolates. The measurement resulted in the values of the three parameters of these isolates, showing that the Kral-3 isolate obtained the highest value, followed by Kral-15, Kral-25, Kral-6, Kral-29, Kral-18, and Kral-4 isolates. During bacterial growth, the biomass increases and affects carotene production and the total carotenoid concentration of isolate. The highest bacterial cell biomass was obtained in the Kral-3 isolate at 7.8 g/L, followed by Kral-15, Kral-25, Kral-6, Kral-29, Kral-18, and Kral-4 isolates at 6.9, 3.6, 2.9, 1.8, 1.4, and

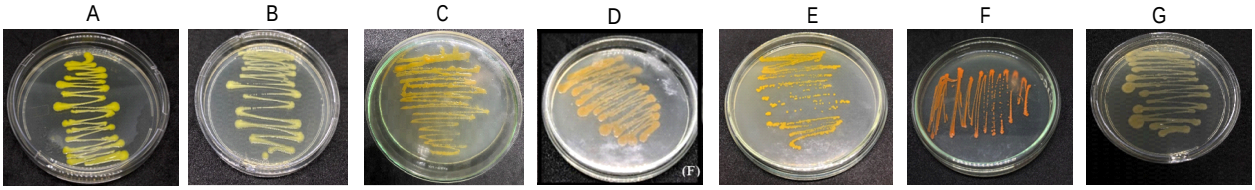

Figure 1. Pigmented heterotrophic bacteria from seawater in Krakal coastal area, Yogyakarta, Indonesia: A. Kral-3 isolate, B. Kral-4 isolate, C. Kral-15 isolate, D. Kral-16 isolate, E. Kral-18 isolate, F. Kral-25 isolate, G. Kral-29 isolate.

Table 1. Colony and cell morphology characteristics of pigmented heterotrophic bacteria isolated from seawater in Krakal coastal area, Yogyakarta

Isolate	Pigment	Morphology of colony			Internal structure	Morphology of cell	
		Shape	Elevation	Margin		Shape	Gram staining
Kral-3	Bright yellow	Circular	Convex	Entire	Smooth	Coccus	Positive
Kral-4	Pale yellow	Circular	Flat	Entire	Smooth	Coccobacillus	Negative
Kral-15	Dark yellow	Circular	Convex	Entire	Smooth	Coccus	Positive
Kral-16	Yellow brown	Circular	Low convex	Undulate	Smooth	Spiral	Negative
Kral-18	Yellow orange	Irregular	Umbonate	Undulate	Rough	Coccobacillus	Positive
Kral-25	Orange	Circular	Convex	Entire	Smooth	Coccobacillus	Positive
Kral-29	Cream	Circular	Convex	Entire	Smooth	Coccus	Positive

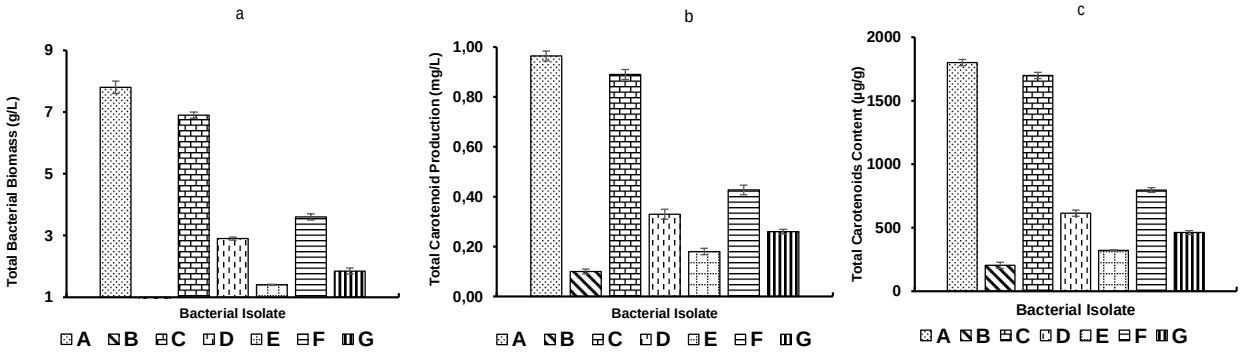

Figure 2. a. Biomass; b. carotenoid production, and c. total carotenoid content of pigmented heterotrophic bacteria from seawater in Krakal coastal area, Yogyakarta, Indonesia, after 144 h of incubation: A. Kral-3 isolate, B. Kral-4 isolate, C. Kral-15 isolate, D. Kral-16 isolate, E. Kral-18 isolate, F. Kral-25.

0.5 g/L, respectively ($p < 0.05$). The highest carotene production after 144 h of incubation was observed in the Kral-4 isolate at 0.96 mg/L, followed by Kral-15, Kral-25, Kral-6, Kral-29, Kral-18, and Kral-4 isolates at 0.89, 0.4, 0.3, 0.26, 0.18, and 0.1 mg/L, respectively ($p < 0.05$). On the contrary, the highest concentration of total carotenoids was produced by the Kral-3 isolate at 1800 µg/g, followed by Kral-15, Kral-25, Kral-6, Kral-29, Kral-18, and Kral-4 isolates at 1698.9, 797.2, 614.8, 462.1, 321.7, and 204.9 µg/g, respectively ($p < 0.05$).

Comparing the measured values of the Kral-4 isolate to those of earlier research, which also produced more significant findings, revealed that it had the highest biomass, carotenoid production, and total carotenoid content. *Formosa* sp. KMW produced total biomass of 6.28 g/L, carotenoid production of 0.977 mg/L, and total carotenoid content of 1260 µg/g biomass after six days of incubation (Sowmya & Sachindra, 2015). By contrast, *Brevundimonas scallop* could produce a total carotenoid content of 1303.62 µg/g biomass (Liu et al., 2020).

Carotenoids produced by these heterotrophic bacteria are associated with protecting cells from UV radiation, which is relatively high in the marine environment (Rodríguez-Concepción et al., 2018; Lozano et al., 2020). UV rays can be classified as UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm). UVA rays will cause the formation of reactive oxygen species in cells, resulting in damage to lipids, proteins, and nucleic acids of bacterial cells. Meanwhile, UVB and UVC rays will directly damage bacterial cell DNA, resulting in photoproducts such as cyclobutane pyrimidine dimers and pyrimidine photoaddition (Styczynski et al., 2020). Several researchers have studied the structure and function of carotenoids as antioxidants, thereby protecting bacterial cells from the harmful effects of UV radiation (Kirti et al., 2014; Mendes-Silva et al., 2021).

Carotenoid extract profiles produced by heterotrophic bacterial isolates based on UV-Vis spectra are presented in Table 2. Carotenoid extracts from Kral-3, Kral-4, and Kral-15 bacterial isolates showed three peaks at UV-Vis readings of 428, 454, and 482 nm. Carotenoid extracts isolated from Kral-16, Kral-18, and Kral-29 showed one peak at a UV-Vis reading of 480 nm. Meanwhile, the carotenoid extract of the Kral-25 isolate showed three peaks at UV-Vis readings of 439, 461, and 491 nm. The difference in the maximum absorption wavelength of the carotenoid extract indicates the different types of carotenoids produced by bacterial isolates. According to Lichtenthaler and Buschmann (2001) and Orona-navar et al. (2017), carotenoid extracts from bacterial isolates Kral-3, Kral-4, and Kral-15 were categorized as zeaxanthin. Carotenoid extracts from bacterial isolates Kral-16, Kral-18, and Kral-29 were categorized as astaxanthin, whereas those from Kral-25 were categorized as γ-carotene.

Table 2. Carotenoid profile of pigmented heterotrophic bacterial isolates based on the UV–vis spectrum

Isolate	Pigment	UV–Vis spectrum (λ_{max} nm)	Carotenoid type
Kral-3	Bright yellow	428, 454, 482	Zeaxanthin
Kral-4	Pale yellow	428, 454, 482	Zeaxanthin
Kral-15	Dark yellow	428, 454, 482	Zeaxanthin
Kral-16	Yellow brown	480	Astaxanthin
Kral-18	Yellow orange	480	Astaxanthin
Kral-25	Orange	439, 461, 491	γ-carotene
Kral-29	Cream	480	Astaxanthin

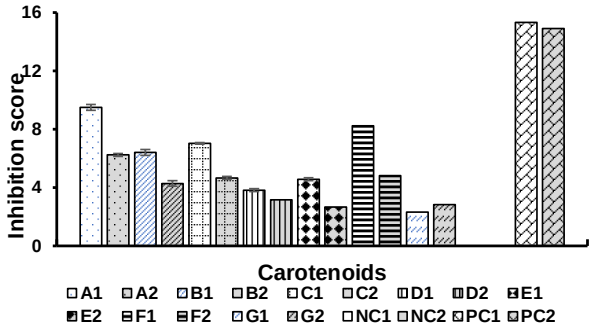

Figure 3. Inhibition score of carotenoid extracts from bacterial isolates against (1) *Escherichia coli* and (2) *Staphylococcus aureus*: A. Kral-3 isolate, B. Kral-4 isolate, C. Kral-15 isolate, D. Kral-16 isolate, E. Kral-18 isolate, F. Kral-25 isolate, G. Kral-29 isolate, NG: Negative control, PC: Positive control

Antibacterial Activity of Carotenoid Extracts from Heterotrophic Bacteria

The measurement results of the antibacterial activity of carotenoid extracts against pathogenic bacteria *E. coli* and *S. aureus* based on the IS are shown in Figure 3. On the contrary, the inhibition zone around the well of carotenoid extracts from bacterial isolates on agar plates against both pathogenic bacteria is shown in Figure 4. The highest IS of 9.5 against pathogenic *E. coli* was measured in the carotenoid extract of the Kral-3 isolate, followed by Kral-25, Kral-15, Kral-4, Kral-18, Kral-16, and Kral-29 isolates at 8.25, 7.04, 6.42, 4.58, and 3.83, respectively ($p < 0.05$). Meanwhile, the highest IS of 6.25 against the pathogenic bacteria *S. aureus* was measured in the carotenoid extract of the Kral-3 isolate, followed by Kral-25, Kral-15, Kral-4, Kral-16, Kral-29, and Kral-18 isolates at 4.83, 4.67, 4.29, 3.17, 2.83, and 2.67, respectively ($p < 0.05$).

According to Tremonte et al. (2017), the IS of the carotenoid extract from Kral-3, Kral-15, and Kral-25 isolates against *E. coli* could be categorized as very strong. In contrast, the carotenoid extract from the Kral-4 isolate was categorized as strong. The carotenoid extracts of other isolates, including Kral-18 and Kral-16, were categorized as moderate, whereas that of the Kral-29 isolate was categorized as low. The IS of the carotenoid extract of the Kral-3 isolate against *S. aureus* was categorized as strong, whereas that of Kral-4, Kral-15, Kral-16, and Kral-25 isolates was categorized as moderate. In addition, the IS of carotenoid extracts of Kral-18 and Kral-29 isolates against *S. aureus* was

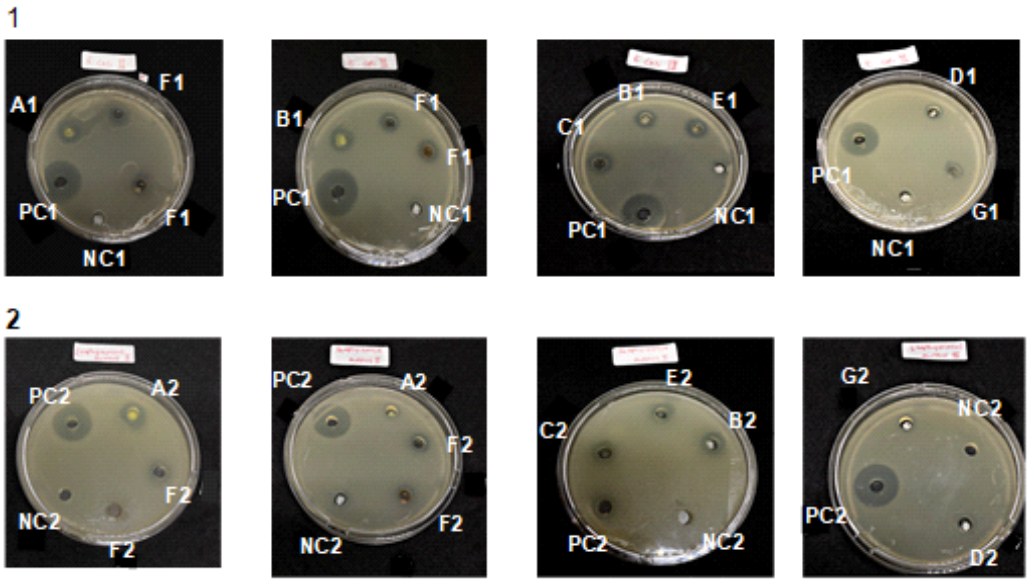

Figure 4. Inhibition zone around the well of carotenoid extracts from bacterial isolates on agar plates against (1) *Escherichia coli* and (2) *Staphylococcus aureus*: A. Kral-3 isolate, B. Kral-4 isolate, C. Kral-15 isolate, D. Kral-16 isolate, E. Kral-18 isolate, F. Kral-25 isolate, G. Kral-29 isolate, NG: Negative control, PC: Positive control

categorized as low. Gökalsýn et al. (2017) reported that the mechanism of the antibacterial activity of carotenoid extracts could occur by inhibiting quorum sensing of pathogenic bacteria, resulting in decreased virulence expression. Paluch et al. (2020) also reported a similar inhibitory mechanism, namely, the inhibition of biofilm formation of pathogenic bacteria through blocking quorum sensing signaling.

Similar researchers also reported the antibacterial activity of astaxanthin pigment extracts from the psychrotrophic bacteria species *Sphingomonas faeni* ISY and *Rhodococcus fascians* CS4 to inhibit the growth of pathogenic bacteria such as *E. coli*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Listeria monocytogenes*, and *V. fischeri* (Mageswari et al., 2015). *Arthrobacter gandavensis* MH187869 was also reported to produce a yellow pigment with antibacterial activity against *K. pneumoniae*, *Enterobacter sakazakii*, and *Salmonella typhi*. Based on GCMS, the bacterial pigment contained six compounds: α-thujene, phosphorous acid, glycerol, 2-butene-1-ol-3-methyl-acetate, camphor, and terpinene-4-ol. On the contrary, based on high-performance liquid chromatography analysis, the bacterial pigment contained 14 different compounds: gallic acid derivative, neoxanthin, capsorubin, carotenoid P468, violaxanthin, gallic acid derivative, monogalloylhexoside, ellagitannin, all-transviolaxanthin, β-carotene, caffeic acid derivative, quercetin-3-*O*-rutinoside 7-*O*-glucoside, bis-HHDP-glucose, and ε-carotene (Numan et al., 2018). Moreover, *V. owensii* TNKJ.CR.24-7 isolated from coral samples produced ε-carotene, which inhibited ESBL *E. coli*, *K. pneumoniae*, and methicillin-resistant *S. aureus* strain (Sibero et al., 2019).

Characteristics of the 16S rRNA Gene in Pigmented Heterotrophic Bacteria

The characterization of the PCR product of the 16S rRNA gene of selected bacterial isolates, namely, Kral-3, Kral-15, and Kral-25, is shown in Figure 5. The selection of bacterial isolates against *E. coli* and *P. aeruginosa* was based on high total carotenoid content and IS of carotenoid extracts, ranging from very strong to moderate. The PCR products of each isolate were detected as DNA fragments of 1500 bp using 1%

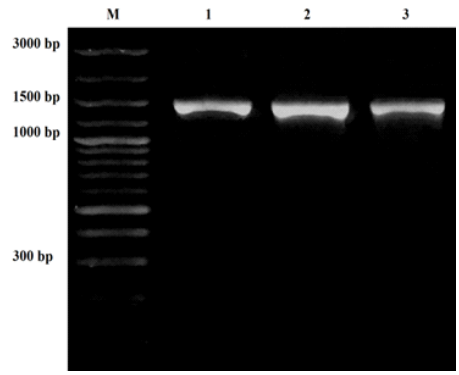

Figure 5. Results for PCR amplification performed using primers 27F and 1492R. (M): Marker DNA 100–300 bp; (1). Kral-3, (2). Kral-15, and (3). Kral-25 isolates of pigmented heterotrophic bacteria from seawater in Krakal coastal area, Yogyakarta, Indonesia.

Table 3. Sequences of pigmented heterotrophic bacterial isolates from seawater in Krakal coastal area, Yogyakarta, Indonesia, based on GenBank data identified using BLASTn

Isolate	Accession number	Species of pigmented bacteria homolog	Identity (%)	Query cover
Kral-3	OM585539.1	*Kocuria rhizophilla strain LSH32*	99.93	100
	KU922406.1	*Kocuria rhizophila strain l49*	99.93	100
	EU554435.1	*Kocuria rhizophilla strain Ag09*	99.93	100
	MN072929.1	*Kocuria varians strain RPLON10*	99.79	100
	NR_026452.1	*Kocurian rhizophilla strain TA68*	99.86	100
Kral-15	EF187228	*Calidifontibacter indicus strain PC IW02*	98.90	99
	KU517232	*Yimmella lutea strain YIM 45900(T)*	97.57	97
	KU881047	*Yimella radicis py1292(T)*	97.39	100
	FJ528304	*Calidifontibacter terrae strain R161(T)*	96.95	100
Kral-25	KY006126.1	*Rhodococcus ruber strain 2S3R1(R)*	99.64	99
	KF803583.1	*Rhodococcus rhodochrous strain KG-21*	99.64	99
	MG190707.1	*Rhodococcus ruber IMB 16-083*	99.35	100
	MZ276291.1	*Rhodococcus electrodiphilus strain JC435*	99.28	100
	MW485814.1	*Rhodococcus ruber strain Y14*	99.28	100
	KC887934.1	*Actinobacterium K15*	99.28	100

agarose gel electrophoresis (Sidin & Retnaningrum, 2022).

The 16S rRNA gene sequences obtained using Sanger sequencing were then compared with databases at GenBank (www.ncbi.nlm.nih.gov and www.ezbiocloud.net). Based on the Basic Local Alignment Search Tool Nucleotide (BLASTn) results, the significant similarity was observed among databases, with similarity values in the range of 99.93%–96.95% (Table 3). Based on such 16S rRNA gene sequences, the Kral-3 isolate showed similarities with *Kocuria rhizophilla* LSH32, with a similarity index of 99.93%. Kral-15 showed similarities with *Calidifontibacter indicus* PC IW02 B, with a similarity index of 98.90%. Kral-29 revealed similarities with *Rhodococcus ruber* 2S3R1, with a similarity index of 99.64% (Sidin & Retnaningrum, 2022). According to Schlaberg et al. (2012), bacteria were classified into the same species when similarity values were >99%. Bacteria were assigned to the same genus for similarity values ranging from 97% to <99%. Furthermore, bacteria were classified into the same family when similarity values ranged from 95% to <97%.

In the present study, phylogenetic analysis was performed using the maximum likelihood method based on 16S rRNA gene sequences of bacteria, showing the formation of two clades (Figure 6). Clade 1, with a bootstrap value of 99, consists of the order Micrococcales, and the second clade consists of the order Mycobacteriales. The first clade has two subclades: the first subclade has a bootstrap value of 100, which consists of the genus Kocuria belonging to the family Micrococcacea, whereas the other subclade consists of the genera *Calidifontibacter* and *Yimella*, which belonged to the family Dermacoccaceae (Sidin & Retnaningrum, 2022). Dahal et al. (2017) also reported that the difference in phenotypic properties between the genera *Califontibacter* and *Yimella* lies in the fatty acids that make up the cells. The second clade with a bootstrap value of 100 consisted of the genus *Rhodococcus* belonging to the family Nocardiaceae. Meanwhile, *V. alginolyticus* strain ATCC 17749 was selected as the outgroup in this analysis. This phylogenetic analysis result of Kral-3, Kral 15, and Kral 25 isolates was in concordance with the findings of BLASTn, where identity scores of 99.93%, 98.90%, and 99.64% were recorded for *K. rhizophilla*, *Calidifontibacter* sp., and *R. ruber*, respectively.

The three pigmented heterotrophic bacterial isolates identified in the species *K. rhizophilla* Kral-3, *Calidifontibacter* sp. Kral-15, and *R. ruber* Kral-25 belonged to the phylum Actinobacteria. Several previous researchers also reported that the members of the phylum Actinobacteria isolated from marine environments produced a large number of secondary metabolites containing bioactive compounds in the form of pigment compounds, including carotene, melanin, phenazine, pyrrole, violacein, quinone, curcumin, and quercetin (Gopikrishnan et al., 2017; Hasan et al., 2020; Kumaran et al., 2020; Velmurugan et al., 2020).

Kocuria spp. belongs to the family Micrococcaceae. The member of this genus has been isolated from various marine, freshwater, dust, and soil environments (Uzair et al., 2018; Mendes-Silva et al., 2021; Wu et al., 2022). The genus *Kocuria* has been studied and reported to produce antibacterial, anticancer, and antioxidant pigments. Monika and Archana (2017) reported that the pigment extract of *K. turfensis* could

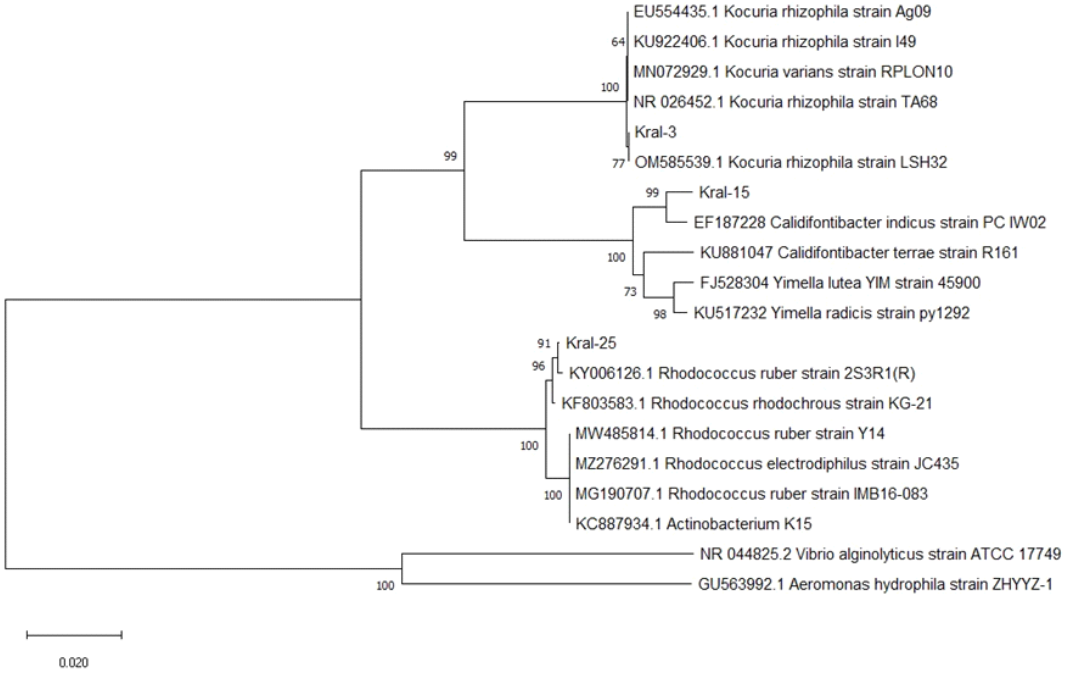

Figure 6. Phylogenetic tree showing the relationship between three pigmented heterotrophic bacterial isolates and 16S rRNA gene sequences obtained from NCBI and EzBioCloud using maximum likelihood analysis with 1000 bootstrap replication, *Vibrio algnolyticus* ATCC 17749 was selected as an outgroup.

inhibit the growth of pathogenic bacteria *E. coli* and *S. aureus*. Other researchers investigated the pigment produced by *K. palustris* HEBS1 showing antibacterial activity against *E. coli* (Sipriyadi et al., 2021). Furthermore, Rezaeeyan et al. (2017) reported that *Kocuria* sp. QWT-12 produces a yellow carotenoid neurosporene that could inhibit cancer cells. Brahma and Dutta (2022) reported the ability of *K. marina* DAGII to produce a carotenoid pigment, namely, beta-cryptoxanthin, which is an antioxidant.

The genus Rhodococcus is also reported to produce bioactive components in the form of carotenoid pigments, antibiotics, siderophores, bioremediator compounds, and biocatalysts (Cappelletti et al., 2020). Several types of carotenoids, *Rhodococcus* spp., have been identified in the form of lycopene, β-carotene, chlorobacter, and astaxanthin compounds and reported to inhibit the growth of several pathogenic bacteria (Styczynski et al., 2020; Velmurugan et al., 2020). Recent research conducted by Çobanoðlu and Yazýcý (2022) reported that carotenoid pigments from isolates of *Rhodococcus* sp. SC1 had antibacterial activity against *E. feacalis* and *S. aureus*. In addition, these pigments inhibit the biofilm formation of pathogenic bacteria *E. coli* and *P. aeruginosa*.

Ruckmani et al. (2011) succeeded in isolating *Calidifontibacter* spp. from hot springs of the Western Ghats, India. In addition, Dahal et al. (2017) reported the ability of the pigment *C. indicus* PC to inhibit the growth of *P. acnes* and *S. epidermidis* bacteria that cause acne, showing their potential to be developed in the cosmetic industry.

The ability of the seven isolates to produce carotenoids with pathogenic antibacterial properties is probably associated with their adaptation to the marine environment caused by high solar radiation. A stressful environment can lead to the accumulation of carotenoids as a cellular defense response. An appealing alternative for specific uses is provided by carotenoids, which are produced by salt-tolerant marine microorganisms. These carotenoids may be used in industrial procedures that utilize substances containing a large amount of salt even after processing. Moreover, seawater is chemically similar to human blood plasma and naturally contains significant salt levels. Therefore, when used for therapeutic purposes, carotenoids made by marine bacteria may be safe, which have no toxicity or decreased toxicity.

Conclusion

This study identified seven heterotrophic bacterial isolates producing carotenoids from seawater in the Krakal coastal area, Yogyakarta, Indonesia. Three

bacteria, namely, Kral-3, Kral-15, and Kral-25, could produce high carotenoids of 1800, 1698.9, and 797.2 μg/g. Based on the antibacterial test using agar well diffusion, the carotenoid extract isolates Kral-3, Kral-15, and Kral-25 showed a very strong IS against *E. coli*. In addition, the carotenoid extract of the Kral-3 isolate showed a strong IS against *S. aureus*, whereas the Kral-15 and Kral-25 isolates had moderate levels. Based on the 16S rRNA gene characterization, Kral-3, Kral-15, and Kral-25 isolates were identified as *Kocuria rhizophilla*, *Calidifontibacter* sp., and *Rhodococcus ruber*, respectively. These three bacterial strains were highly developed as bioproducts in the pharmaceutical and biotechnology industries.

Supplementary Material

Supplementary material is not available for this article.

References

Ambati, R. R., Phang, S. M., Ravi, S., & Aswathanarayana, R. G. (2014). Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications-A review. *Marine Drugs*, *12*(1), 128-152. https://doi.org/10.3390/md12010128.

Aryee, A. N., Agyei, D., & Akanbi, T. O. (2018). Recovery and utilization of seaweed pigments in food processing. *Current Opinion in Food Science*, *19*, 113-119. https://doi.org/101016/j.cofs.2018.03.013.

Asker, D. (2018). High throughput screening and profiling of high-value carotenoids from a wide diversity of bacteria in surface seawater. *Food Chemistry*, *261*, 103-111. https://doi.org/10.1016/j.foodchem.2018.03.109.

Asker, D., Awad, T. S., Beppu, T., & Ueda, K. (2018). Rapid and selective screening method for isolation and identification of carotenoid-producing bacteria. *Methods in Molecular Biology, 1852*, 143-170. https://doi.org/10.1007/978-1-4939-8742-9_9.

Boontosaeng, T., Nimrat, S., & Vuthiphandchai, V. (2016). Pigments production of bacteria isolated from dried seafood and capability to inhibit microbial pathogens. *IOSR Journal of Environmental Science*, *10*(5), 30-34. https://doi.org/10.9790/2402-1005023034.

Brahma, D., & Dutta, D. (2022). Antioxidant property of beta-cryptoxanthin produced by *Kocuria marina* DAGII. *Materials Today: Proceedings*, 57, 1833-1837. https://doi.org/10.1016/j.matpr.2022.01.027.

Cappelletti, M., Presentato, A., Piacenza, E., Firrincieli, A., Turner, R. J., & Zannoni, D. (2020). Biotechnology of Rhodococcus for the production of valuable compounds. *Applied Microbiology and Biotechnology, 104*(20), 8567-8594. https://doi.org/10.1007/s00253-020-10861-z.

Çobanoðlu, Þ., & Yazýcý, A. (2021). Isolation, characterization, and antibiofilm activity of pigments synthesized by *Rhodococcus* sp. SC1. *Current Microbiology*, *79*(1), 1-10. https://doi.org/10.1007/s00284-021-02694-4.

Dahal, R. H., Shim, D. S., Kim, J. Y., & Kim, J. (2017). *Calidifontibacter terrae* sp. nov., an actinomycete isolated from soil, with potential applications in cosmetics. *International Journal of Systematic and Evolutionary Microbiology*, *67*(6), 1925-1931. https://doi.org/10.1099/ijsem.0.001893

Esatbeyoglu, T., & Rimbach, G. (2017). Canthaxanthin: From molecule to function. *Molecular Nutrition and Food Research*, *61*(6), 1-7. https://doi.org/10.1002/mnfr.201600469.

Gökalsýn, B., Aksoydan, B., Erman, B., & Sesal, N. C. (2017). Reducing virulence and biofilm of *Pseudomonas aeruginosa* by potential quorum sensing inhibitor carotenoid: Zeaxanthin. *Microbial Ecology*, *74*(2), 466-473. https://doi.org/10.1007/s00248-017-0949-3.

Gopikrishnan, V., Radhakrishnan, M., Pazhanimurugan, R., Shanmugasundaram, T., & Balagurunathan, R. (2017). Antimicrobial, antitubercular and antiproliferative activities of quercetin isolated from the marine *Streptomyces fradiae*. Bangladesh, *Journal of Pharmacology, 12*(3), 333-334. https://doi.org/10.3329/BJP.V12I3.33208.

Hasan, M., Elkhoury, K., Belhaj, N., Kahn, C., Tamayol, A., Barberi-Heyob, M., Arab-Tehrany, E., & Linder, M. (2020). Growth-inhibitory effect of chitosan-coated liposomes encapsulating curcumin on MCF-7 breast cancer cells. *Marine Drugs* 18(4). https://doi.org/10.3390/md18040217.

Kirti, K., Amita, S., Priti, S., Mukesh Kumar, A., & Jyoti, S. (2014). Colorful world of microbes: Carotenoids and their applications. *Advances in Biology*, *2014*, 1-13. https://doi.org/10.1155/2014/837891.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura K. (2018). MEGA X molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547-1549. https://doi.org/10.1093/molbev/msy096.

Kumaran, S., Bharathi, S., Uttra, V., Thirunavukkarasu, R., Nainangu, P., Krishnan, V. G., Renuga, P. S., Wilson, A., & Balaraman, D. (2020). Bioactive metabolites produced from *Streptomyces enissocaesilis* SSASC10 against fish pathogens. *Biocatalysis and Agricultural Biotechnology*, *29*, 101802. https://doi.org/10.1016/j.bcab.2020.101802.

Liang, J., Liu, J., Zhan, Y., Zhou, S., Xue, C. X., Sun, C., Lin, Y., Luo, C., Wang, X., & Zhang, X. H. (2021). Succession of marine bacteria in response to *Ulva prolifera*-derived dissolved organic matter. *Environment International*, 155, 106687. https://doi.org/10.1016/j.envint.2021.106687.

Lichtenthaler, H. K., & Buschmann, C. (2001). Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. *Current Protocol in Food Analitycal Chemistry*, *1*(1), F4.3.1–F4.3.8. https://doi.org/10.1002/0471142913.faf0403s01.

Liu, H., Zhang, C., Zhang, X., Tan, K., Zhang, H., Cheng, D., Ye, T., Li, S., Ma, H., & Zheng, A. (2020). A novel carotenoids-producing marine bacterium from noble scallop *Chlamys nobilis* and antioxidant active ties of its carotenoid compositions. *Food Chemistry*, 320, 126629. https://doi.org/10.1016/j.foodchem.2020.126629.

Lozano, C., Matallana-Surget, S., Givens, J., Nouet, S., Arbuckle, L., Lambert, Z., & Lebaron, P. (2020). Toxicity of UV filters on marine bacteria: Combined effects with damaging solar radiation. *Science of the Total Environment*, *722*, 137803. https://doi.org/10.1016/j.scitotenv.2020.137803.

Mageswari, A., Subramanian, P., Srinivasan, R., Karthikeyan, S., & Gothandam, K. M. (2015). Astaxanthin from psychrotrophic *Sphingomonas faeni* exhibits antagonism against food-spoilage bacteria at low temperatures. *Microbiological Research*, 179, 38-44. http://doi.org/10.1016/j.micres.2015.06.010

Mendes-Silva, T. C. D., Vidal, E. E., de Souza, R. F. R., Schmidt, K. C., Mendes, P. V. D., da Silva Andrade, R. F., da Silva Oliveira, F. G., de Lucena, B. T. L., de Oliveira, M. B. M., dos Santos Correia, M. T., & da Silva, M. V. (2021). Production of carotenoid sarcinaxanthin by *Kocuria palustris* isolated from Northeastern Brazil Caatinga soil and their antioxidant and photoprotective activities. *Electronic Journal of Biotechnology*, *53*, 44-53. https://doi.org/10.1016/j.ejbt.2021.05.004.

Mohana, D., Thippeswamy, S., & Abhishek, R. (2013). Antioxidant, antibacterial, and ultraviolet-protective properties of carotenoids isolated from *Micrococcu*s spp. *Radiation Protection and Environment*, *36*(4), 168. https://doi.org/10.4103/0972-0464.142394

Monika, R. T., & Archana, P. S. (2017). In vitro antagonistic activity of chromobacteria against human pathogenic bacteria. *International Journal for Researches in Biosciences, Agriculture and Technology*, *5*(2), 1-19.

Narsing Rao, M. P., Xiao, M., & Li, W. J. (2017). Fungal and bacterial pigments: Secondary metabolites with wide applications. *Frontiers in Microbiology*, *8*, 1-13. https://doi.org/10.3389/fmicb.2017.01113.

Numan, M., Bashir, S., Mumtaz, R., Tayyab, S., Ullah, I., Khan, A. L., Shinwari, Z. K., & Al-Harrasi, A. (2018). Chemical profile and in-vitro pharmacological activities of yellow pigment extracted from *Arthrobacter gandavensis*. *Process Biochemistry*, 75, 74.82. https://doi.org/10.1016/j.procbio.2018.08.033.

Orona-Navar, A., Aguilar-Hernández, I., Cerdán-Pasarán, A., López-Luke, T., Rodríguez-Delgado, M., Cárdenas-Chávez, D. L., Cepeda-Pérez, E., & Ornelas-Soto, N. (2017). Astaxanthin from *Haematococcus pluvialis* as a natural photosensitizer for dye-sensitized solar cell. *Algal Research*, *26*, 15-24. https://doi.org/10.1016/j.algal.2017.06.027.

Paluch, E., Rewak-Soroczyñska, J., Jêdrusik, I., Mazurkiewicz, E., & Jermakow, K. (2020). Prevention of biofilm formation by quorum quenching, *Applied Microbiology and Biotechnology***,** *104*(5), 1871-1881. https://doi.org/10.1007/s00253-020-10349-w.

Ravikumar, S., Uma, G., & Gokulakrishnan, R. (2016). Antibacterial property of halobacterial carotenoids against human bacterial pathogens. *Journal of Scientific and Industrial Research*, *75*(4), 253-257.

Rezaeeyan, Z., Safarpour, A., Amoozegar, M. A., Babavalian, H., & Tebyanian, H. (2017). Original article: High carotenoid production by a halotolerant bacterium, *Kocuria* sp. strain QWT-12 and anticancer, *EXCLI Journal Experimental and Clinical Sciences*, *16*, 840-851. https://doi.org/10.17179/excli2017-218.

Rodríguez-Concepción, M., Avalos, J., Bonet, M. L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, D., Limon, M. C., Meléndez-Martínez, A. J., Olmedilla-Alonso, B., Palou, A., Ribot, J., Rodrigo, M. J., Zacarias, L., & Zhu, C. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Progress in Lipid Research*, *70*, 62-93. https://doi.org/10.1016/j.plipres.2018.04.004.

Ruckmani, A., Kaur, I., Schumann, P., Klenk, H. P., & Mayilraj, S. (2011). *Calidifontibacter indicus* gen. nov., sp. nov., a member of the family dermacoccaceae isolated from a hot spring, and emended description of the family dermacoccaceae. *International Journal of Systematic and Evolutionary Microbiology, 61*(10), 2419-2424. https://doi.org/10.1099/ijs.0.025593-0.

Sanger, F., Nicklen, S., & Coulson, R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA*, *74*(12), 5463-5467 https://doi.org/https://doi.org/10.1073/pnas.74.12.5463

Schlaberg, R., Simmon, K. E., & Fisher, M. A. (2012). A systematic approach for discovering novel, clinically relevant bacteria. *Emerging Infectious Diseases*, *18*(3), 422-430. https://doi.org/10.3201/eid1803.111481.

Sibero, M. T., Bachtiarini, T. U., Agus Trianto, A., Lupita, A. H., Sari, D. P., Igarashi, Y., Harunari, E., Sharma, A. R., Radjasa, O. K., & Sabdono, A. (2019). Characterization of a yellow pigmented coral-associated bacterium exhibiting antibacterial activity against multidrug resistant (MDR) organism. *Egyptian Journal of Aquatic Research*, *45*(1), 81-87. https://doi.org/10.1016/j.ejar.2018.11.007

Sidin, R. S., & Retnaningrum, E. (2022). Karakterisasi bakteri penghasil pigmen dari Pantai Krakal, Gunung Kidul, Yogyakarta dan potensinya sebagai antibakteri. *Final Research Project of Graduate Student*. Universitas Gadjah Mada, Yogyakarta, Indonesia.

Sinha, S., Choubey, P., Kumar, A., & Bhosale, P. (2017). Research article identification, characterization of pigment producing bacteria from soil and water and testing of antimicrobial activity of bacterial pigments. *International Journal of Pharmaceutical Sciences Review and Research*, *42*(23), 119-124.

Sipriyadi, Cahlia, U., Darwis, W., Wibowo, R. H., Nugraheni, E., & Sariyanti, M. (2021). Identification bacteria associated with Haliclona sp. sponges from Enggano Island, Indonesia with antimicrobial activity against human pathogens. *Malaysian Journal of Microbiology*, *17*(2), 178-189. https://doi.org/10.21161/mjm.200843.

Sowmya, R., & Sachindra, N. M. (2015). Carotenoid production by Formosa sp. KMW, a marine bacteria of Flavoba cteriaceae family: Influence of culture conditions and nutrient composition. *Biocatalysis and Agricultural Biotechnology*, *4*(1), 559-567. http://doi.org/10.1016/j.bcab.2015.08.018.

Srinivas, T. N. R., Sudharani, P., Behera, S., Dutta, S., Kumar, G. S., Sathibabu, Y., Geethika, G., Swagata, C., & Kandar, P. P. (2021). Influence of anthropogenic activities on the spatial distribution of heterotrophic, indicator and pathogenic bacteria around Andaman and Nicobar Islands, *Marine Pollution Bulletin*. *162*, 111839. https://doi.org/10.1016/j.marpolbul.2020.111839

Styczynski, M., Rogowska, A., Gieczewska, K., Garstka, M., Szakiel, A., & Dziewit, L. (2020). Genome-based insights into the production of carotenoids by Antarctic bacteria, *Plannococcus* sp. ANT_H30 and *Rhododcoccus* sp. ANT_H53B. *Molecules, 2(*4357), 1-17. https://doi.org/doi:103390/molecules25194357.

Thanapimmetha, A., Suwaleerat, T., Saisriyoot, M., Chisti, Y., & Srinophakun, P. (2017). Production of carotenoids and lipids by *Rhodococcus opacus* PD630 in batch and fed-batch culture. *Bioprocess and Biosystems Engineering, 40*(1), 133-143. https://doi.org/10.1007/s00449-016-1681-y.

Tremonte, P., Pannella, G., Succi, M., Tripaldi, L., Sturchio, M., Coppola, R., Luongo, D., & Sorrentino, E. (2017). Antimicrobial activity of *Lactobacillus plantarum* strains isolated from different environment: A preliminary study. *International Food Research Journal*, *24*(2), 852-859.

Usman, H. M., Abdulkadir, N., Gani, M., & Maiturare, H. M. (2017). Bacterial pigments and its significance. *MOJ Bioequivalence & Bioavailability, 4*(3), 285-288. https://doi.org/10.15406/mojbb.2017.04.00073.

Uzair, B., Menaa, F., Khan, B. A., Mohammad, F. V., Ahmad, V. U., Djeribi, R. & Menaa, B. (2018). Isolation, purification, structural elucidation and antimicrobial activities of kocumarin, a novel antibiotic isolated from actinobacterium *Kocuria marina* CMG S2 associated with the brown seaweed *Pelvetia canaliculata*. *Microbiological Research, 206*, 186-197. http://doi.org/10.1016/j.micres. 2017. 10. 007.

Velmurugan, P., Venil, C. K., Veera Ravi, A., & Dufossé, L. (2020). Marine bacteria is the cell factory to produce bioactive pigments: A prospective pigment source in the ocean. *Frontiers in Sustainable Food Systems, 4*, 1-7. https://doi.org/10.3389/fsufs.2020.589655.

Wu, Z., Lyu, H., Ma, X., Ren, G., Song, J., Jing, X. & Liu, Y. (2022). Comparative effects of environmental factors on bacterial communities in two types of indoor dust: Potential risks to university students. *Environmental Research, 203*,111869. https://doi.org/10.1016/j.envres.2021.111869.

Zhao, Y., Guo, L., Xia, Y., Zhuang, X., & Chu, W. (2019). Isolation, identification of carotenoid-producing Rhodotorula sp. from marine environment and optimization for carotenoid production. *Marine Drugs, 17*, 161. https://doi.org/10.3390/md17030161.