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

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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 (polysoprene) 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. pyogenes, 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. xylosox, Peanibacillus macerans, and Citrobacter diversus (Boontosaeng et al., 2016). Sibero et al. (2019) have isolated Vibrio owensii TNKJ.CR.24-7 from coral samples of Karimunjawa National Park, Jepara Region, Central Java Province, Indonesia. V. owensii TNKJ.CR.24-7 produced β-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°08′ 46.23 S 110°35′ 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):

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

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 10⁶ 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 ≤ IS < 5, strong if 5 ≤ IS < 7, and very strong if 7 ≤ 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 GGYTACCTTGTAGTGACTT-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

Colonies, 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-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
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-16, 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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/g biomass after six days of incubation (Sowmya & Sachindra, 2015). By contrast, *Brevundimonas scallop* could produce a total carotenoid content of 1303.62 \(\mu\)g/g biomass (Liu et al., 2020).

| Isolate | Pigment          | Morphology of colony | Morphology of cell |
|---------|------------------|----------------------|-------------------|
|         |                  | Shape    | Elevation | Margin | Internal structure | Shape | Gram staining |
| Kral-3  | Bright yellow    | Circular | Convex   | Entire  | Smooth            | Coccos | 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 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.

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.
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). UV 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 Lichtenhaler and Buschmann (2001) and Orona-navar et al. (2017), carotenoid extracts from bacterial isolates Kral-3, Kral-16, Kral-18, and Kral-29 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 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 (\(\lambda_{\text{max}}\) 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-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-methylacetate, 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.](image)
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 Micrococcaceae, 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 *Calidifontibacter* 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. rhizophila*, *Calidifontibacter* sp., and *R. ruber*, respectively.

The three pigmented heterotrophic bacterial isolates identified in the species *K. rhizophila* 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

| Isolate      | Accession number | Species of pigmented bacteria homolog | Identity (%) | Query cover |
|--------------|------------------|--------------------------------------|--------------|-------------|
| Kral-3       | OMS85539.1       | Kocuria rhizophilla strain LSH32      | 99.93        | 100         |
|              | KU922406.1       | Kocuria rhizophilla strain 49         | 99.93        | 100         |
|              | EU554435.1       | Kocuria rhizophilla strain 499        | 99.93        | 100         |
|              | MN072929.1       | Kocuria varians strain 499           | 99.79        | 100         |
|              | NR_026452.1      | Kocuria rhizophilla strain TA68       | 99.86        | 100         |
| Kral-15      | EF187228         | Calidifontibacter indicus strain PC IW02 | 98.90    | 99          |
|              | KU517232         | Yimella lutea strain YM 45900(T)      | 97.57        | 97          |
|              | KU881047         | Yimella radicis py1292(T)            | 97.39        | 100         |
|              | FJ528304         | Calidifontibacter tereae 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          |
|              | MGJ90707.1       | Rhodococcus ruber strain IMB 16-083  | 99.35        | 100         |
|              | MZZ76291.1       | Rhodococcus electrophilas strain JC435 | 99.28   | 100         |
|              | MW485814.1       | Rhodococcus ruber strain Y14          | 99.28        | 100         |
|              | KCR87934.1       | Actinobacterium K15                   | 99.28        | 100         |

The 16S rRNA gene sequences obtained using Sanger sequencing were then compared with databases at GenBank (www.ncbi.nlm.nih.gov and www.ebiocloud.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%.

Table 3. Sequences of pigmented heterotrophic bacterial isolates from seawater in Krakal coastal area, Yogyakarta, Indonesia, based on GenBank data identified using BLASTn.
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, beta-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 Çobanoğlu and Yazıcı (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 Escherichia coli. In addition, the carotenoid extract of the Kral-3 isolate showed a strong IS against Staphylococcus 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.

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