Exploring bioactive pigments from marine bacterial isolate from the Indonesian seas

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Abstract. Marine microbes offer a significant source for biodiscovery due to their rich biodiversity and genetic capacity. Particularly, microbial pigments of marine origin are getting more attention in current research due to their widely perceived application as natural food colorants, antioxidant, antimicrobial, and many more. In the past five years, our research group has successfully characterised various bioactive pigments isolated from marine bacteria, including *Erythrobacter flavus* strain KJ5 that produces unique sulphur-containing carotenoids *Pseudoalteromonas rubra* strain PS1 and SB14 that contain antimicrobial prodiginine, and *Seonamhaeicola algicola* strain CCI for high content of zeaxanthin. This paper describes the challenges we encountered in conducting research in exploring bioactive pigments especially with focus on carotenoid research, reviewed critically on strategy we developed for isolation of isolate as well as identification and elucidation of the pigments, and consideration for future research.

Keywords: bioactive pigments, carotenoid, mass spectrometry, UV-vis spectrum

1. Introduction
Bioactive pigments isolated from marine microbes are currently being explored for clinical and industrial applications because they demonstrated various activity such as antioxidant, anticancer, antimicrobial, as well as acting in stimulating immunity [1]. Bioactive pigments, such as violacein (purple), quinone (bright yellow), phenazines (yellow crystalline), prodiginines (red), and carotenoids (yellow to red) can be easily detected by naked eye from the gram-positive and gram-negative bacteria after incubation on common microbial cultures media containing seawater-rich [2]. A current review on the recent exploration of bioactive pigments from marine bacteria reported that there was an increasing number of articles reporting on bacterial pigments from only 8 article in 2002 to 84 articles by 2015, moreover in the past five years at least 17 new species have been successfully isolated from their native habitat [3].

A common marine environment to discover novel bacterial isolate producing bioactive pigments is symbiosis or “the living together” interaction between marine invertebrate, especially the sessile ones as hosts and microorganisms as symbionts [4]. Microbial symbionts have been witnessed to produce a variety of tailored biochemical traits, due to co-evolution with their specific host, making them a rich source of secondary metabolites with properties of medically and commercially attractive bioactivities[5-7]. Carotenoid has been considered as a bioactive compound of the highest market demand, because of their biotechnological application and their potential beneficial uses in human
healthcare, food processing, pharmaceuticals, and cosmetics [7]. Carotenoids are a family of yellow to orange-red pigments with the structure composed of mainly C40 lipophilic isoprenoids. Carotenoids have important biological roles in all living systems. In addition to their function to promote photosynthesis process, another relevant function of carotenoid is associated with their properties as antioxidant, scavenger of free radicals and protection against blue light radiation, which also correspond to their molecular structure [8]. There are more than 250 naturally occurring carotenoids of marine origin had been reported as of 2017 [9]. The different compositions that are present in the marine organisms has promoted the use of carotenoids as a chemical signature for rapid chemotaxonomic profiling [10-12]. Carotenoid producing bacterial isolate that are associated to marine invertebrate reported from 1994 to 2019 is presented in Table 1, in which at least four species had been discovered from the Indonesian seas.

Table 1. Carotenoid producing marine bacterial isolates that are associated to coral and sponges as hosts, reported from 1994 to 2019 searched through Science Direct and Pubmed.

| Location of Discovery | Host                  | Type of Carotenoid                  | Characterization Technique         | Ref.  |
|----------------------|-----------------------|------------------------------------|------------------------------------|-------|
| Gaetbulimicrobium sp. strain 04OKA-17-12 | Kerama Islands, Japan | Acropora nobilis | Zeaxanthin | UV vis, HPLC, MS, NMR | [13] |
| Virgibacillus salarius CBSCP 1-1 | Karimun Jawa Island, Indonesia | Sarcophyton sp. | β-carotene | UV-Vis HPLC | [14] |
| Pseudoalteromonas shioyasakensis CBSCP 2-2 | Karimun Jawa Island, Indonesia | Sarcophyton sp. | β-carotene | UV-Vis HPLC | [14] |
| Erythrobacter flavus strain KJ5 | Karimun Jawa Island, Indonesia | Acropora nasuta | Nostoxanthin sulfate, Caloxanthin sulfate, Zeaxanthin sulfate, Nostoxanthin, Caloxanthin, Zeaxanthin, B-cryptoxanthin, β-carotene | UV-Vis HPLC, FT-IR, MS | [15] |
| Micrococcus yunnanensis | Akajima Island, Japan | Unidentified hard coral | Sarcinaxanthin, Sarcinaxanthin monoglucoside, Sarcinaxanthin diglucoside | UV-Vis HPLC, MS, NMR | [16] |
| Vibrio owensii TNKJ.CR.24-7 | Karimun Jawa Island, Indonesia | Unidentified coral | β-carotene | UV-Vis, TLC | [17] |
| Flexibacter sp. strains DK30213 | Sagami Bay, Japan | Raniera japonica  | Zeaxanthin | TLC, UV-Vis HPLC, MS | [18] |
| Pseudomonas sp. strain KK10206C | Suruga Bay, Japan | Halichondria okadaï | Okadaxanthin | UV-Vis, CD, MS, NMR | [19] |
| Rubritalea spongiae strain YM21-132T | Natsudomari Peninsula, Japan | Unidentified sponge | Unspecified carotenoid | HPLC, MS | [20] |
| Flavobacterium sp. | Palau | Homaxinella sp. | Myxol | UV Vis, HPLC, MS, NMR | [21] |
| Location of Discovery | Host | Type of Carotenoid | Characterization Technique | Ref. |
|-----------------------|------|-------------------|--------------------------|------|
| Rubritalea squalenifaciens | Miura peninsula, Japan | Halichondria okdai | Diapolycedopenioic acid xylosyl ester A, Diapolycedopenioic acid xylosyl ester B, Diapolycedopenioic acid xylosyl ester C | [22, 23] |
| Sphingomonas sp. KODA19-6 | Kood Island, Thailand | Tethya seychellensis | Zeaxanthin | [24] |
| Algoriphagus sp. KK10202C | Numazu, Japan | Halichondria okdai | Flexixanthin, Deoxyflexixanthin | [25] |
| Aquimarina spongiae | Jeju Island, South Korea | Halichondria oshoro | Unspecified carotenoid | [26] |
| Psychrobacter celer strain CBSP5 | Karimunjawa Island, Indonesia | Callyspongia vaginalis | Unspecified carotenoid | [27] |
| Streptomyces strain AQBWWS1 | Kovalam Coast, India | Callyspongia diffusa | Lycopene | [28] |
| Salegentibacter agarivorans | Onecotan Island | Artemisina sp | Unspecified carotenoid | [29] |
| Formosa spongicola | Jeju Island, South Korea | Hymeniacidon flavia | Unspecified carotenoid | [30] |
| Algoriphagus sp. strain KK10202C | Numazu, Japan | Halichondria okdai | lycopene, 3, 4-dehydrodorhopin, deoxyflexixanthin, flexixanthin | [25] |

2. Methodology

2.1. Challenges and strategy in getting the isolate

It is extremely challenging to get microbes from the seawater sample that can readily cultured and sustainably grow under laboratory condition. Although rapid progress in development of cultivation technique has been demonstrated and reported to result in the successful isolation of high number of novel microorganisms [31]. In most cases we frequently encountered situations where we have an insufficient knowledge of the microbes themselves, and the organic substrates that they use in the sea. This is dilemmatic and there is a circularity. It is a kind of serendipity to design targeted research to obtain promising isolate that produce bioactive compounds. An advantage of working with pigment-producing marine bacteria is that we can easily distinguish the different colony by its first sight (Figure 1). When using spot spectrophotometer such as USB4000 spectrophotometer from ocean optics, the typical spectra of the pigments each colony can be recorded.

Coral reefs are excellent sources for finding bacterial isolate with potential novel carotenoid with novel properties. In the case of corals and sponges, the associated diversity of microbial is known to be influenced by host interaction, production of antimicrobial compounds, and environmental conditions [33]. Interestingly, Symbionidium dinoflagellates, members of the coral holobiont that do photosynthesis, also influence microbial community structure by releasing complex carbon-containing exudates including dimethylsulfiniopropionate (DMSP) [34]. DMSP is a stable organosulfur compound derived from sulfur-containing amino acid and DMSP a key compound in the global sulfur cycle. It can be degraded to dimethylsulphide (DMS), which is very important in climate regulation owing to its role in cloud formation [34]. However, some coral such as Acropora spp. also produces DMSP even in the
absence of Symbiodiniaceae symbionts [34]. In addition to DMSP, isoprene is also known emitted by endosymbiont of reef-building corals [35]. Isoprene is produced from dimethylallyl diphosphate (DMAPP), a precursor molecule for many building blocks of cells, including sterols, quinones, hormones, chlorophylls, and carotenoids. Coral-associated bacteria have potential roles in carbon, nitrogen, and sulfur, and give protection to host [36], therefore it is interesting to find new bacterial isolate that demonstrate such capacity, for example bacteria that can synthesis sulfated natural product compounds, particularly carotenoid pigments. In the past five year, we succeeded to discover from Acropora nasuta a yellowish marine bacterium Erythrobacter flavus strain KJ5, which capable to metabolize sulfate into the carotenoid structure [15, 37].

![Figure 1](image-url)

**Figure 1.** Bacterial colonies first appearing on Zobell agar medium appear to produce an array of pigments: (a) Seawater sample collected from Sebanjar Beach, Alor Island, (b) Seawater sample collected from Kondang Merak Beach, South Malang, East Java, (c) Pure colonies of Pseudoalteromonas rubra that produces antibacterial red pigment prodigiosin [32], (d) Pure colonies of Seonamhaeicola algicola strain CC1 that produce high concentration of zeaxanthin carotenoid. Note: not all isolated colony was survived for long period of purification and for a sustainable culture in the laboratory condition.

2.2. **Screening strategy of carotenoid-producing bacteria**

The first step to identify whether the coloured colonies containing carotenoid is by measuring their UV-vis spectrum of the pigments. This can be done either by direct measurement on the colony using a portable spectrophotometer or by extraction of pigment using organic solvent and then measure the UV-vis spectrum of the crude extract. A typical UV-vis absorption spectrum of carotenoid is characterised by a strong broad band with peaks or shoulders between 400 to 550 nm. Some carotenoid even shows prominent three characteristic absorption peaks when dissolved in non-polar solvents. In the second step involves sequancing of 16S rRNA genes of the potential candidates. Sequencing and phylogenetic analysis of the potential carotenoid producing bacterial isolates are needed to find the closest species with known carotenoid composition from the carotenoid database (http://carotenoiddb.jp/). In many cases, unique carotenoids are not available commercially. Therefore, it is very important to have a selection of bacteria whose carotenoid types can be used as a reference in the next stage of analysis.

NCBI BLAST is the most used sequence similarity search tool. If the determined sequence of an unknown isolate exhibits high similarity, between 97% to 100% with the reference sequenced of an approved species, then the unknown isolate may be assigned to this species. Deep literature research then needs to be carried out to find out whether any pigments analysis has been conducted. In our case, we succeeded to find the gap in the reported information about Pseudoalteromonas rubra and we prove that it produces antibacterial prodigiosin pigment [32]. When the similarity of sequence is between 97% and 95%, the unknown species can be assigned to the corresponding genus with possibility of claiming a novel species. In this case, based on our experience it is worth to conduct pigment analysis thoroughly because the unknown species might contain other type of carotenoid although it has the same biosynthetic pathway as it is reported. In the case of Erythrobacter flavus strain KJ5, for example, we
even conducted a whole genome sequencing prior to build a hypothesis of the possibility to find a new type of sulphate containing carotenoid [37]. To analysis the carotenoid composition, we used high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and triple quadrupole mass spectrometry (MS/MS), because we already have identified the carotenoids from the approved species as reference compounds. When the similarity is less than 95%, the unknown isolate can be assigned to a family with possibility of claiming a novel genus and with a high chance to find novel carotenoids. In the later case, one might need a high-resolution mass spectrometry and a nuclear magnetic resonance (NMR) in addition to optical spectroscopy for the elucidation of the carotenoid structure.

3. Results and discussion

3.1. HPLC-ESI-MS/MS for the analysis of carotenoids

In comparison to the thin layer chromatography (TLC), nowadays, HPLC has been used on daily bases for separation and identification of carotenoids because of many reasons, including large selection on the separating columns, ease modification of mobile phase composition, possibility to alter the peak separation by tuning the column oven temperature, and lots of choices for the type of detectors. Gas chromatography (GC) is not suitable because carotenoids have low volatility and thermostability [38]. HPLC can be coupled with DAD to provide data consists of the separation profile and the UV-vis absorption spectra of each separated components. HPLC can be also coupled with MS system to provide additional data about the molecular weight of the carotenoid.

In our screening approach, we usually use the following HPLC method. A reversed phase (RP) C$_8$ column with two eluents as mobile phase. Eluent A is composed of methanol, acetonitrile, and pyridine solution with pH is maintained at pH 5. Eluent B is composed of methanol, acetonitrile, and acetone. We use flow rate 1 mL/min and let the detection time finished after 40 minutes. It is quite a slow method, however, our method has been succeeded to resolve the separation of unique carotenoids that usually overlapping with other components [15, 39]. To confirm the result obtained using the C$_8$ column, we usually carry out experiment with a YMC Carotenoid column, which is powerful to give high resolution with a clear separation of xanthophyll cycle carotenoids [40]. For the method using a YMC Carotenoid column, we use a gradient elution program with the eluent composes of water, methanol, and methyl tert-butyl ether (MTBE).

In most successful cases for carotenoid studies, electrospray (ESI) and atmospheric pressure chemical ionization (APCI) are well compatible ionization techniques with LC-MS. Carotenes and xanthophylls form molecular ions or protonated molecules during positive ion electrospray, however the hydrocarbon carotenes do not ionize when using the negative mode. When using APCI, abundant positively or negatively charged molecular ions or protonated and deprotonated molecules are formed for both carotenes and xanthophylls [41]. However, it depends on the purpose of structural elucidation and the availability of the ionization module. In our laboratory, unfortunately we only have ESI. In this case, we don’t care much on the hydrocarbon carotenes. We are interested more in the end groups which carotenoids have a lot of variation (see and compare different carotenoid structures that are available in the carotenoid database, http://carotenoiddb.jp/). For this purpose, it is important to activate the ESI in the positive and negative mode. For example, the detection of nostoxanthin sulfate from E. flavus strain KJ5 was effectively observed in the negative mode (Figure 2).

Nostoxanthin (C$_{50}$H$_{56}$O$_{10}$), has the exact mass of 600.42 u, shows a molecular ion at m/z 600.5 [M] with a product ion at m/z 508.6 [M – 92], which corresponds to a loss of toluene [15]. However, nostoxanthin sulfate cannot be detected in the positive mode of Q1 scan (Figure 2). When detected in the negative mode of Q1 scan, the molecular ion appears at m/z 679.3 [M-H-Na] with high signal intensity. The product ion scan of nostoxanthin sulfate in the negative mode consistently produces a product ion at m/z 97.3, which indicate sulfate ion [HSO$_4$] well recorded by electrospray ionization. The intensity of m/z 679.3 also shows consistent decrease proportionally with the increase of the intensity of m/z 97.3 at higher collision energy.
Figure 2. ESI-MS/MS analysis of the nostoxanthin sulfate in *E. flavus* strain KJ5, full Q1 scan at the positive and negative mode, and product ion scans (right) spectra using collision energy (CE) at 15 and 30 V. Adapted from Setiyono et al. 2019 [15].

3.2. Consideration for future research

Many organisms tend to accumulate carotenoid under prolong biotic and abiotic stresses through upregulation of carotenoid biosynthetic pathways in response to avoid degradation [42, 43]. During thermal stress, photodamage, photoinhibition and increasing of cellular respiration in *Symbiodinium*, for example, result in the production of reactive oxygen species (ROS) at the chloroplast, mitochondria and microbody [44]. The accumulation of carotenoid is a perfect scenario to overcome the excess of ROS in the cell. Therefore, the search of bioactive pigments, especially carotenoids and other similar antioxidant compounds, that is also related to the local environmental changes of these marine bacterial isolates [3] might be interesting to be considered and evaluated for the future research.

4. Conclusion

Research in the exploration of bioactive pigments has gain an increasing interest despite challenges in cultivation of microbes from the seawater sample in a sustainable manner under laboratory condition. A strategy to obtain unique bioactive pigments includes exploring of microbial community in the coral reefs. The screening strategy for determination of novel carotenoids in marine microbes includes UV-vis spectrum analysis of the pigment crude extract, genome analysis and subsequently HPLC-MS/MS analysis. The results of genomic analysis that have been compared with the database will produce references to nearby species that have similar carotenoid compositions. The use of HPLC-MS/MS analysis can provide a reference regarding the exact composition and content of carotenoids.

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