Characterization of Tambjamines Pigment from Marine Bacterium Pseudoalteromonas sp. PM2 Indigenous from Alor Island, Indonesia

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INTRODUCTION

More than 70% of the earth’s surface is marine and theoretically marine has greater biodiversity than land [1]. In the last decade, the research on the natural compounds from marine become attractive research rather than research on the land. Marine organisms have unique secondary metabolites due to the high pressure in marine environments like high salinity and temperature, as well as low nutrition. Those conditions drive them to produce secondary metabolites as a self-defense mechanism.

The produced pigment is one of the famous secondary metabolites from marine [2]. Besides that, pigments have bioactive properties that can be applied in the pharmacological industries such as anticancer, antibacterial, antioxidant, antimalarial, antifungal, anti-inflammatory, food dye, and other functions [3]. The utilization of natural pigments from marine organisms should consider the sustainability of marine resources. Marine microorganisms such as bacteria could become an alternative source of natural pigment from marine and not damaging the marine ecosystems. Another advantage is they are easy to be cultured in a laboratory. Hence, the research to find natural pigments from bacteria will always become an interesting object for the researcher.

At least, there are seven kinds of pigment that can be found from marine bacteria. There are prodiginines, carotenes, violacein, phenazine, quinones, tambjamines, and melanins. Other pigments, scytomenin (produced by aquatic Cyanobacteria) and yellow tryptanthrin (from marine bacterium strain AM13, the genus of Flexibacteria) were not belong to seven pigments mentioned above [2]. Prodiginines are the red pigment firstly isolated from Bacillus prodigious. Although, now the name of Serratia marcescens was used to renamed Bacillus prodigious [4]. In our previous study, six types of prodigine found in marine bacterium Pseudoalteromonas (P.) rubra PS1 and they exhibited antimicrobial activity [5].

Carotenoids, a yellow or orange to red pigment, found in plants, animals, and microorganisms (bacterium and...
Setiyono, Se locations, i.e., Sika Island coast (A), Mali Beach coast (B), Tenggara, Indonesia (8°15'S and 124°45'E). Samples of marine bacteria from four sampling sites as on Alor Island, Indonesia. The culturable strains were dominated by yellow pigmented bacteria such as Streptomyces sp. B6921 and Streptomyces sp. M045 [13–15]. Tambjamines are yellow pigment isolated from P. tunicata, have biological activity as antifouling, anti-microorganisms, anti-invertebrate larvae, antialgal spores, anti-protozoan, and antifungal, and play a role as protector for host marine organisms [16,17]. Melanins pigments are produced by some marine bacterium such as Vibrio cholerae, Shewanella colwelliana, and Alteromonas nigrifaciens [18–21]. A melanin derivative, phaeomelanin which synthesized by Cellulophaga tyrosinolyxans has been known to have tyrosinase activity [22]. Pigments from marine bacteria not only give color to the bodies of these bacteria, but their extensive applications properties have been attracted scientists to examine them.

Hence, we interested to find the source of marine pigmented bacteria which can produce pigments continuously. Recently, we have successfully isolated six marine-pigmented bacteria from four sampling sites as on Alor Island, Indonesia. The culturable strains were dominated by yellow-pigmented marine bacteria that produced pigments with the same spectral UV-Vis indicated that might they were the same species. Here, we reported the characterization of their pigment according to spectrophotometer UV−Vis and FTIR, as well as separation by reverse-phase high performance liquid chromatography. We also identified the strain molecularly by 16S rRNA gene sequencing.

EXPERIMENTAL

General

The bacterial cells was purified in microbiology laboratorium of Ma Chung Research Center for Photosynthetic Pigments. Extraction of tambjamines was conducted under controlled condition. Methanolic extract of tambjamines was investigated using spectrophotometer UV-Vis, reverse phase HPLC and FTIR.

Samples Collection, Bacterial Isolation and Purification

Sampling was conducted on Alor Island, East Nusa Tenggara, Indonesia (8°15’S and 124°45’E). Samples of seawater were collected by diving from four different locations, i.e., Sika Island coast (A), Mali Beach coast (B), Sebanjar Beach coast (C), and Alor Kecil Beach coast (D) at the surface and bottom layer of locations (Figure 1). The sample then put on a sterile tube (50 mL) and placed in a cold box completely with ice to keep the temperature. Samples were brought to Ma Chung Research Center for Photosynthetic Pigments rapidly. A total of 1 mL of seawater sample was mixed into 9 mL of sterile seawater and homogeneous with a vortex to obtain dilution 1 or 10⁻¹. A total of 1 mL of seawater from dilution 1 is taken and 9 mL of sterile seawater was added and homogenized to obtain dilution 2 or 10⁻². Dilution was carried out up to 6x with the same process. From each dilution, 35 μL of seawater was taken and spread directly to a sterile petri dish containing marine media agar (MA) 2216E (Difco, USA) and incubated for 3 days at 32 °C. MA media are used because they contain macro and micronutrients specifically for the culture of marine bacteria. After 3 days of incubation, bacterial colonies on petri dishes were observed. Colored bacterial colonies were purified by the streak plate method and cultured in a new medium for 3 days at 32 °C. Purification of pigmented bacteria was based on their color, shape, margins, height, and size. Purification is carried out repeatedly until the colony is pure. Pure bacteria were then stored on glycerol-containing media at 10 °C until used.

Bacterial Culture and Pigment Extraction

After obtaining pure bacteria, bacterial cells were mass-cultured in MA media in the same culture period. The determination of bacterial cell harvest time was based on the growth curve. Cells were harvested when they reached the final exponential phase, approaching the stationary phase. In this phase, pigment production already reached its highest peak production. The bacterial cells were harvested directly from the agar medium by taking it using a sterile one and placing it in a 10 mL eppendorf bottle. The cell yields are stored in a freezer at −35 °C until used. The pigments were extracted using methanol and acetone (7:3, v/v). 1 mL solvent was used to extract 0.1 g bacterial cells. CaCO₃ and sodium ascorbate were added to the mixture to prevent pigment degradation by the oxidation process. The mixture bacterium cells and solvents were homogenized by a vortex for 5 times (1 min vortex, 1 min on ice) and then crushed by sonication at a pulse mode with 60% amplitude and 10 s off for 10 min (QSonica, Newtown, State, USA). The cell debris and supernatant were separated by centrifugation at 10,000 rpm for 3 min. The supernatant extract was collected and dried using a rotary evaporator (100 rpm, 35 °C on water, 5 °C temperature of chiller). The dried pigments extract was stored at −30 °C prior to be used.

Identification of Pigment by Spectrophotometer UV-Vis

Spectrophotometer UV−Vis (Spectrophotometer UV−Vis 1700 Shimadzu, Japan) was used to determine the spectra of the tambjamine crude extracts. Previously, methanol was used as a background before measurement. The dried tambjamine extracts were diluted in methanol and measured at the wavelength (λ) 200–1,100 nm. Subsequently, the obtained data were analyzed using software OriginPro 8.5.1 (OriginLab, Northampton, Massachusetts, USA). Identification pigments based on spectral properties and maximum wavelength absorbance (λmax) and study comparative with references.

Identification of Pigment by FTIR

The dried tambjamines extract were analyzed by using Fourier transform infrared spectroscopy (FTIR). Measurements were conducted with a Jasco FTIR-6800 (Tokyo, Japan), using the ATR (Diamond) method. The type of ATR used was the...
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Isolated strains were obtained from different sampling locations that have a different ecosystem from each to others. Sika Island is the living place for Dugong dugon dominated by the seagrass ecosystem. The Mali Beach ecosystem is composed of sand and dead coral. Whereas other locations, Sebanjar and Alor Kecil beach is an area with a good coral reef ecosystem. One yellow strain, PS2, was obtained from Sika Beach from the surface of seawater. In Mali Beach, one yellow strain, PM2 was also obtained and isolated from the surface of seawater. Whereas from Sebanjar Beach, two yellow strains, SB11 and SB13 were isolated either from the surface and bottom layer of location with 6 m depth of the bottom area. On the other hand, we also found two strains from Alor Kecil Beach also from the surface and bottom area of seawater (Figure 2).

Identification of Pigment by FTIR

FTIR analysis was conducted to confirm the structural identification of tambjamine form. Figure 4 shows the FTIR spectra of the crude tambjamine extract from marine bacterium strain PM2 which showed absorption at 3310, 2951, 2844, 2372, 2320, 1639, 1116, 1014, and 881 cm⁻¹. Absorption at 3310 cm⁻¹ indicates O-H and N-H stretching from the solvent (MeOH) and amine groups of tambjamines. Absorption at 2951 and 2844 cm⁻¹ reveal sp³ and sp² C-H stretching, respectively. Atmospheric CO₂ contributes to weak absorption at 2372 and 2320 cm⁻¹. C=N and C-N stretching from tambjamines molecule are displayed at 1639 and 1116 cm⁻¹ absorption, respectively. The presence of ether group (C-O) in tambjamines is shown at 1014 cm⁻¹ absorption. Besides, C-H bending gives absorption at 881 cm⁻¹. The results are in line as reported by [30-33]. In nature, there are 14 types of tambjamines. However, their basic structure is characterized by a 2-methoxy-2’,3’-bipyrralenamine [34]. Tambjamines consists of two pyrrole rings with an enamine moiety at C-5 and a methoxy group at C-4. Most have short alkyl chains substituted for the enamine nitrogen [33]. Some members of tambjamines such as tambjamine B, D, G, H, I, and J also bind bromine.
HPLC Analysis

Crude tambjamines extracts all strains were separated and analyzed through RP–HPLC by using a C4 column. Chromatogram HPLC of six strain showed the same pattern. For further analysis, we chosen crude tambjamines extract from strain PM2. At least there are five different tambjamines types that have been separated clearly. The most polar compound 1 is eluted at tR 2.24 min and shows λmax at 374 nm. The second compound 2 that slightly more non-polar than 1 is the major compound eluted at tR 2.56 min with λmax at 376 nm. Whereas the last three compounds 3, 4, 5 have tR at 2.84, 3.68, 3.99 min and λmax at 379, 391, and 392 nm, respectively (Figure 5). The absorption spectra of five compounds are characterized by a broadband in the range λ 300–500 nm with an absorption maximum varied at 374–392 nm. So far, there are 14 types natural tambjamines, i.e., A, B, C, D, E, F, G, H, I, J, K, YP1, MYP1, and BE-18591 [17,29–33,35–36]. They absorb wavelength in the range of 300–500 nm in methanol. Tambjamine A and B have λmax at 397 nm. Tambjamine C, D, E, and F have λmax at 401–407 nm. Tambjamine K has λmax at 412 and 455 nm. Whereas, tambjamine MYP1 and BE-18591 show λmax at 385 and 368 nm, respectively. There is no information λmax for tambjamine G, H, I, J, and YP1 until now. Among them, YP1 and MYP1 are synthesized by marine bacterium from genus Pseudoalteromonas while tambjamine BE-18591 is produced by land Streptomyces.

Molecular Identification of Bacteria by 16S rRNA Sequence Analysis

Analysis of yellow pigment by using spectrophotometer UV−Vis, FTIR, and HPLC revealed that there was no different signal among six isolated strains. It indicates that six strains might be the same species. Therefore, strain PM2 was selected for molecular identification by 16S rRNA gene sequencing. The molecular analysis of the 16S rRNA gene sequence of strain PM2 consists of 1351 bp. The nucleotide data has been registered to the DNA Data Bank of Japan with the accession number LC505058. The 16S rRNA gene similarity of PM2 was determined in the NCBI nucleotide BLAST. Based on the similarity search, strain PM2 was closely related to P. piscicida strain NBRC 103038 with homology 99.63%. In the phylogenetic constructed with the neighbor-joining tree, strain PM2 joined the type strain of P. piscicida strain NBRC 103038 with a bootstrap resampling value of 100% (Figure 6). PM2 and P. piscicida strain NBRC 103038 was also joined in the tree constructed with the maximum-likelihood and maximum-parsimony algorithm and the recovered nodes were marked by an asterisk in Figure 6 below. P. piscicida is a yellow pigmented-marine bacterium isolated from estuarine waters. It has been reported can produce a neuromuscular toxin which able to kill numerous fish and crab species. Nevertheless, until now, the compound has never been confirmed and identified [37–39]. The closest possibility, it was a yellow cyclic/acyclic brominated depsipeptide compounds [28].

Figure 3. Absorption spectra of crude pigment extract in methanol. The yellow pigment from all strains showed the same typical absorption spectra with λmax at 386–387 nm.

Figure 4. FTIR spectra of crude tambjamines extract from marine bacterium strain PM2

Initial identification by spectra UV−Vis, FTIR, and RP–HPLC revealed that yellow pigment produced by our isolated strains was tambjamines. Until now, only two marine bacteria, i.e. P. citrea and P. tunicata have been known to synthesize tambjamine. Our strain, PM2, also produce tambjamine and belong to genus Pseudoalteromonas. The other members within this genus, P. aurantia [40], P. luteoviolacea [28], P. maricoloris [40], P. peptidolytica [41], and P. piscicida [42], produce yellow pigment. However, the pigment has never been identified. Our new finding indicated that in the group of marine bacteria, tambjamine might be only synthesized by members from genus Pseudoalteromonas.
CONCLUSIONS

Six isolated strains of marine bacteria have been isolated from four different sampling location in Alor Island, Indonesia. Characterization of crude extract pigment was conducted using spectrophotometer UV-Vis, HPLC, and FTIR. All strains produced tambjamines pigment according to UV-Vis and FTIR spectra data. Selected strain, PM2, has been identified based on 16S rRNA gene sequences analysis and belong to genus Pseudoalteromonas. So far, only two marine bacteria have been known to produce tambjamine and they are from genus Pseudoalteromonas. Our new finding indicated that in the group of marine bacteria, tambjamine might be only synthesized by members from genus Pseudoalteromonas. On the other hand, PM2 can be used as source of natural tambjamines

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Abstrak

Pigmen dari bakteri laut telah menarik perhatian bagi ilmuwan karena aplikasinya yang luas dan saat ini eksplorasi sumber pigmen baru dari bakteri laut masih berlangsung. Baru-baru ini kami telah berhasil mengisolasi enam bakteri laut berpigm ungu dari pulau Alor, Indonesia. Spektra UV-Vis dan FTIR ekstrak pigmen kasar dari enam strains menunjukkan karakteristik dari tambjamines, suatu kelompok pigmen kuning yang biasanya ditemukan dalam nudibranchs dan bryozaans. Selain itu, pemisahan dan karakterisasi ekstrak kasar tambjamines menghasilkan lima jenis tambjamine yang berbeda dengan absorbansi maksimum pada panjang gelombang 374–392 nm. Berdasarkan analisis terhadap susunan gen 16S rRNA, strain PM2 hampir se dekat berhubungan terhadap beberapa spesies dalam genus *Pseudoalteromonas*, dengan kemiripan lebih dari 99%. Strain PM2 dirancang sebagai *Pseudoalteromonas* sp. PM2 dengan nomer aksesi LC505058. Sejauh ini hanya dua bakteri laut yang telah diketahui dapat menghasilkan tambjamine, dan mereka berasal dari genus *Pseudoalteromonas*. Penemuan baru ini menunjukkan bahwa dalam kelompok bakteri laut, tambjamine kemungkinan hanya disintesis oleh anggota dari genus *Pseudoalteromonas*.

Kata kunci: pigment, tambjamines, marine bacteria, *Pseudoalteromonas*

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