

Prospective Source of Antimicrobial Compounds From Pigment Produced by Bacteria associated with Brown Alga (Phaeophyceae) Isolated from Karimunjawa island, Indonesia

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Abstract. Brown algae or Phaeophyceae characterized by their natural pigments that differ from other important algal classes. Several publications proves that brown algae - associated bacteria have great potential in developing marine pharmaceutical industry since they are capable to synthesized numerous bioactive metabolite compounds. However the potency of marine pigmented microbes associated with brown alga to produce natural pigments and antimicrobials has been less studied. Marine pigmented bacteria associated with brown algae collected from Karimunjawa Island were successfully isolated and screened for antimicrobial activity. The aim of this research was evaluated of the antimicrobial activity of pigments extracted from culturable marine pigmented bacteria on some pathogenic bacteria and yeast. The results showed that all isolates had antimicrobial activity and could be prospectively developed as antimicrobial agent producing pigments. The 6 marine pigmented bacteria was identified to genus level as *Pseudoalteromonas*, *Sphingomonas*, *Serratia*, *Paracoccus*, *Vibrio*.

Keywords: Marine pigmented bacteria, brown algae, antimicrobial activity, Karimunjawa

1. Introduction

Pigments are widely used in food [1], pharmaceutical [2], textile [3], cosmetics [4]. The need of pigment from natural sources has increased because of awareness of consumers about hazardous of synthetic colors [5]. Therefore, it is imperative to find an alternative pigment which can be biodegraded and easily available at a minimal production cost [4-5]. Carotenoids, one of the main pigments, can be synthesized from plant and microbial sources. Algae are marine photosynthetic organisms, whose pigment profiles are used as a basis for taxonomic classification into green, red and brown algae [6]. Brown algae pigments have been reported that have great pharmaceutical prospects [7-10], nutraceuticals [11] and cosmeceuticals [12-13]. Brown algae and the pigmented bacteria are independently studied. Marine and terrestrial microbes differ from each other due to the influence of their respective environmental conditions. Marine bacteria, is a very interesting organism for researchers because it potentially produces compounds with unique biological properties [14].



The uniqueness of the antimicrobial component produced by marine microbes is influenced by the host species as well as the environmental conditions in which the organism grows. Based on the phenomenon, microbial symbionts, especially the symbionts of brown algae that grow in Karimunjawa Island, is expected to answer the problem of limitations of brown algae use as a source of pigment. Therefore, marine pigmented microbes associated with brown algae having the ability to produce unique antimicrobial components can be an alternative source for exploring marine natural products with promising pigment as a bioactive compounds.

2. Methodology

2.1. Algae sampling

Algae samples (Genera of Sargassum and Padina) were collected from Karimunjawa Island, Jepara Indonesia at 0,5 – 2 m depth into the ocean.. The collected samples were rinsed with sterile seawater and put into sterile ziplock plastic bag labelled contains sterile seawater and were stored in cool box.

2.2. Isolation and purification of marine microbes

Isolation of microbes was carried out by spread and pour plate method using Zobell Marine Agar 2216 media (Hi-Media), Nutrient Agar (Merck), R2A Agar (Oxoid). Approximately 5 g (wet weight) of sample was suspended in 200 mL of sterile seawater in erlenmeyer flask. This was kept in a rotary shaker at 120 rpm for 15 min. [3] with slight modification. Then the water samples were serially diluted and plated in duplicates. Plates were incubated at room temperature for 48 - 168 h. Distinct pigmented colonies were selected and purified. The pure isolate was used for next steps.

2.3. Screening antimicrobial activity

2.3.1. Pigment extraction

The pigment was extracted according to the method of [15-16], with some modifications. The bacterial cells were first grown for 24 - 48 h followed by centrifugation at 7500 rpm for 10 min. The cells in the mixed solution were broken up by adding 5 sterile glass beads (0.4 mm diameter). The suspension was then separated by centrifugation at 5,000 g for 20 minutes at 4⁰C in the ratio of 1: 5 (supernatant) or until the pellet was colorless, i.e., complete pigment extraction has been achieved. The bacterial cell pellet was then discarded, while the supernatant is then dried with N₂ gas. The pigment concentration process was carried out until around 1% (v/v) of the initial solvent volume was left in the evaporation flask. The concentrated pigment was then transferred onto glass Petri dishes prior to drying for 3 days at 25⁰ C. Crude extracts are stored at -18⁰ C until used.

2.3.2 Antimicrobial activity of the extracted pigment

Antimicrobial activities of Extract pigment were studied against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans* obtain from Biotechnology Lab of Biologi Dept Diponegoro University. . Microorganisms were grown in Zobell Marine Broth (for bacteria) and PDB (for yeast) at 28⁰C for overnight. The overnight culture measured equivalent to 0.5 mc Farland standard. The dilution was used as the inoculums for antimicrobial activity assay. 0,1 ml pathogenic bacteria were inoculated by spread method on MA media for bacteria and PDA for yeast. The paper disks contained the carotenoid extract were placed on the agar media surface. Inhibition zones were recorded after overnight incubation at 28 ⁰C [17-18].

2.4. Identification of the isolate

2.4.1. PCR amplification 16S rDNA gene sequence

DNA extraction for bacteria was obtained by chelex method with slight modification [19] Supernatant which contains DNA used as a template for PCR analysis. Primers used for PCR amplification were universal primer 27F(5'-AGAGTTTGATCMTGGCTCAG-3) and 1492 R (5'-GGTTACCTTGTTACGACTT-3') [20]. The PCR mixture consisted of *forward* primer (27 F) 1 µl,

Reverse primer (1492 R) 1 μ l, DNA template 2 μ l, 10 x Buffer 5 μ l, 10 mM dNTP mix 1 μ l, 25 mM MgCl₂ 3 μ l, ddH₂O 34 μ l. The total volume measured 50 μ l. The PCR condition was pre denaturation at 95⁰ C for 3 min, denaturation at 94⁰ C for 1 min, annealing at 54⁰ C for 1 minute, extension at 72⁰ C for 30 second and post cycling at 72⁰ C for 7 minute. All condition were repeated 30 times. The PCR products were loaded in agarose gel (1%) for electrophoresis then visualized by UVI Doc HD5

2.4.2. DNA sequencing

Sequencing of extracted DNA was sent a sequencing service company. The alignment of sequences result were done using MEGA 6, then alignment result was inserted to the BLAST program to create homology of any closely related bacteria in the gene bank database [21]

3. Results and discussion

3.1. Isolation and purification of brown algae pigmented symbiont

Brown algae are considered to be nutrient-rich organisms that trigger high competition among microbial communities [22-24]. Recent studies suggest that microbial communities associated with algae are significantly different from those in seawaters [25-26]. Isolation of pigmented bacteria was grown using 4 types of general media, Zobell Marine Agar, Nutrient Agar, R2A and Potato Dextrose Agar at the first step. Associated microbes were extracted from *Sargassum sp* and *Padina sp* thallus and cultivated on all four media, but on subsequent observations Zobell Marine Agar media provide suport for pigmentation and growth for bacteria.

A total of 36 types of pigmented microbes asociated with brown algae based on morphological colony differences were found in the present study. All isolate were selected based on distinct pigmented colonies, micromorphological characteristics, their ability to grow and the stability of the pigments produced on the media used. Selection results obtained 6 types of bacterial isolates that will be used in the next stage of research. There were 6 brown algae -associated bacteria as shown at Fig 1.

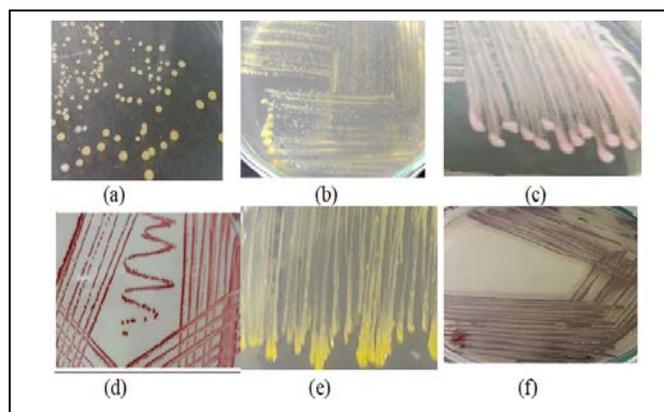


Figure 1. Characteristics and growth of bacteria morphotypes on media Zobell Marine Agar
a – f: Genera isolate bacteria brown algae symbionts.

3.2. Screening of bacteria producing antimicrobial compounds

Brown Algae which are phototrophs, is one of the important members of the marine ecosystem not only individually as a food source, but also at the level of the ecosystem as a habitat-forming and primary producers [26]. Studies on brown algae suggest that brown algae produce bioactive components that exhibit anti-obesity, anti-inflammatory, anti hypertensive, anticholesterol [8] activities to prevent osteoporosis and rheumatoid arthritis [27] and anticoagulant [28]. Symbiotic microbes are known to be

capable of producing the same compounds as their hosts, although they may not be identical to the original components produced by their host. Recent publications report that most of the active compounds isolated from marine organisms, produced by symbiotic microbes [29].

Genera of Sargassum and Padina are known to have antimicrobial activity and are prospective agents for the development of antimicrobial components [30-31]. The screening procedure to investigate antimicrobial activity in this study used microorganisms test of both Gram positive and Gram negative as a target strains, as well as pathogenic yeast. Those microorganism test are *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. Based on medical point of view, these pathogenic test strain get important attention because they are well represented the causative agents for human infection [32].

Based on the results obtained, pigment extracts from bacterial isolates that had previously been observed with a uv vis spectrophotometer were belonged in the carotenoid group. According to the result, most of the carotenoid extract of isolates were able to produce the antimicrobial activity on the all of the microbial test strain shown by the inhibition zone on agar plate. Isolates are more able to inhibit the growth of gram-positive bacteria than gram-negative bacteria. Data presentation refers to research conducted [32] (Table 1).

Table 1. Screening of antimicrobial activity of carotenoid extract brown algae associated bacteria against pathogenic microbes.

NO	Inhibition of test strains	Activity pattern	Number of isolates	Percentage
1	Only <i>E. coli</i>	a	3	50
2	Only <i>S. aureus</i>	b	4	83,3
3	Only <i>B. subtilis</i>	c	6	0,67
4	Only <i>C. albicans</i>	d	4	0,67
5	<i>S. aureus</i> and <i>B. subtilis</i>	e	4	0,67
6	<i>S. aureus</i> and <i>C. albicans</i>	f	3	50
7	<i>B. subtilis</i> and <i>C. albicans</i>	g	3	50
8	<i>E. coli</i> and <i>S. aureus</i>	h	3	50
9	<i>E. coli</i> and <i>B. subtilis</i>	I	3	50
10	<i>E. coli</i> and <i>C. albicans</i>	j	3	50

The result of this research is consistent with previous research done by [32] that revealed the antibiotic activity of bacteria associated with brown algae *Laminaria saccharina* and found that more than 50 % associated bacterial strains inhibited the growth of either gram-negative or gram-positive bacteria or yeast.

Several studies on bacterial symbionts have shown that they have potential to function as natural pigment sources and bioactive compounds. It has been reported that several new pigmented strains of bacteria are explored from various habitats, and have beneficial secondary metabolites, such as: *Exiguobacterium* sp. The yellow pigment-producing bacteria isolated from Marina Beach [33], *Janthinobacterium lividum XT1*, the pigment-producing violet producer of glacier in China [34], a psychophysiological bacteria RT102 suspected to be a *Janthinobacterium* bacteria producing violacein have high anti-bacterial activity and anti protozoa and antifungal [35,36], *Pseudoalteromonas tunicata* marine bacteria producing dark green pigments, potentially as antifouling [37]. Marine bacteria *Pseudoalteromonas piscicida* H1.7 isolated from Kakaban Land-Locked Marine Lake showed antimicrobial activity towards *Staphylococcus aureus* and produced a yellow pigment [38].

Based on the facts mentioned above, reinforced by the results of antimicrobial activity screening has been done. In principle we know that symbiotic microbes are capable of producing prospective secondary metabolite components, but they may be produced by specific conditions as well because the marine environment is a unique environment such as certain salt concentrations, hydrostatic pressures, competition between microbes and nutrients [39] These results are likely to be different if performed on

a laboratory scale, whose is well controlled and generally tries to use microbial cultivation on liquid medium as a main methods and also depend on secondary metabolite product. [40-41]. Although the microbial cultivation method based on fermentation in the laboratory is a promising effort in the discovery of new compounds [41].

3.3. Analysis of PCR products

PCR products (2 μ l) were analyzed by agarose gel electrophoresis. Electrophoresis was performed at 100V for 30 min. The gels were stained for 45 min with gel red nucleic acid gel stain (Biotium) and photographed under UV illumination.

All isolates were successfully amplified by PCR (Figure 2).

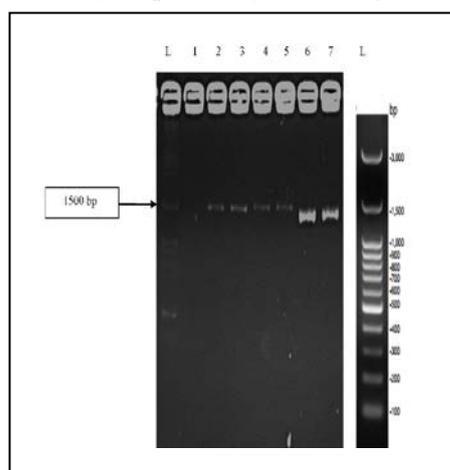


Figure 2. Visualization of PCR product
where: L = Ladder; 1 = negative control 1-7 : isolates

The closest phylogenetic relatives of each isolate were identified by comparison of the 16S rRNA gene sequence to the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tools. Blast homology search result of the potential pigmented microbes was shown in Table 2.

Table 2. Homology of *brown algae pigmented symbiont*

NO	CODE	Closest Relative	E Value	Homology
1	KR11	<i>Pseudoalteromonas flavipulchra strain ES8</i>	0,0	99 %
2	KR124	<i>Sphingomonas desiccabilis Strain CP1D</i>	0,0	99 %
3	KR16	<i>Serratia rubidaea strain N22</i>	0,0	99 %
4	KR19	<i>Serratia rubidaea strain NBRC103169</i>	0,0	97 %
5	KR120	<i>Paracoccus marcusii</i>	0,0	97 %
6	KR121	<i>Vibrio fortis strain 9G1</i>	0,0	97 %

In the recent decades, considerable intensive research on bacterial communities associated with algae has been conducted. It is directed to study bacterial communities associated with algae, an understanding of the succession of community structures, in terms of the interactions between microbes and algae. and

no less important is the exploration of natural bacterial products associated with algae [22][43]. However, based on a comprehensive study with sequences of 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) fingerprinting, it is known that microbial communities associated with algae differ from planktonic bacterial communities [21][43-44].

PCR amplification of 16S rDNA from six bacterial isolates were showed positive results with the presence of appropriate base length of approximately 1300 - 1500 bp (Fig. 1). Brown algae marine pigmented microbial associant that have been isolated are included in the phyla Proteobacteria (Alpha and Gamma). This result is consistent with the study that members of Alphaproteobacteria and Gammaproteobacteria have a more global distribution and are found to be associated with eukaryotic in marine and coastal areas. Meanwhile, other taxa that are also often found are Bacteroidetes, Actinobacteria, Planctomycetes and Chloroflexi [22][46-47]

Genus *Pseudoalteromonas* that reported capable of producing yellow and orange pigment. epiphytic *Pseudoalteromonas tunicata* and *Roseobacter gallaeciensis* also have been found in association with green alga *U. australis* and capable producing a range of extracellular inhibitory compounds against marine fungi, bacteria, invertebrate larvae, and algal spores [48-49]. Bacteria of the genus *Pseudoalteromonas* play an important role in marine environments because of its ability to produce highly variable metabolites including bioactive components and enzymes. These bacteria are spreading cosmopolitan because they can adapt and survive in a poorly nutrient marine environment and extreme pressures [48][50-51]. The study conducted [52] stated that genus *Pseudoalteromonas* is of great interest to the scientific community because of (i) its prolific metabolite-producing capacity and (ii) its usual association with macroorganisms, leading to a suspected and sometimes documented ecological significance. [53] highlighted a *Pseudoalteromonas* isolate as producer of a novel antimicrobial protein, which inhibited human pathogenic strains causing dermatologic diseases.

Studies that have been done also found two types of serratia, namely *S. marcescens* and *S. rubideae*. Characteristics of *Serratia* that successfully isolated is a convex-shaped colonies and produce red pigment. This bacteria is facultative and able to grow well in aerobic and anaerobic conditions in artificial media. The red pigment produced by *S. marcescens* is a secondary metabolite known as prodigiosin from the tripyrrole family which generally contains 4-methoxy, ring 2-2 bipyrrrole ring system [54]. Prodigiosin is a promising drug due to its reported, antibacteria, antifungal, immunosuppressive and anti-proliferative activities [55-56]. in this study, *Serratia* able to inhibit bacteria either from gram positive or gram negative bacteria.

Other pigmented bacteria found in this study were from the genera *Sphingomonas*, *Vibrio* and *Paracoccus*. Genus *sphingomonas* have been isolated from a variety of habitats, such as rhizospheres, soil, aquatic habitats and clinical material, but little is known about *Sphingomonas* strains dwelling in marine environments, particularly associated with animals. The publication of the *Sphingomonas* isolation from the marine that has been successfully done are *Sphingomonas molluscorum* sp. nov., a novel marine isolate associated with marine mollusc *Anadara broughtoni* [57], the crab *Paralithodes camtschatica*, a marine crustacean [58] and *Sphingomonas jejuensis* sp. nov., isolated from marine sponge *Hymeniacidon flavia* [59]. Several representatives of genus *Sphingomonas* have been reported to produce anticyanobacterial compounds such as argimicins and antimicrobial compounds [56].

The genera of pigmented bacteria also found in this study is that of the genus *Paracoccus*. The genus *Paracoccus* consists of Gram-negative, oxidase and catalase-positive, capable of producing astaxanthin and great antimicrobial activity. At present, the genus *Paracoccus* includes 17 recognized species [31][60].

4. Conclusion

Despite the great challenge and difficulty in isolating and harvesting marine pigmented bacteria, especially those bacteria are associated with marine organisms, but the progress of exploration in this field is promising. In particular, based on studies antibacterial activity of pigmented bacteria associated with brown algae and types of isolates that were successfully obtained, the development of bioactive

components of these bacteria can be developed more widely and continue to provide promising avenues for both fundamental sciences and applied biomedical research.

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