Isolation and Characterization of Bacteria Associated with Brown Algae Sargassum spp. from Panjang Island and Their Antibacterial Activities

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Abstract

Bacteria associated with brown algae represent a rich source of bioactive metabolites. Twenty-three marine bacterial strains associated with three species of brown algae Sargassum (S. polycystum, S. duplicatum and S. echinocarphum) were isolated using ZoBell 2216E from Panjang island, Jepara, North Java. The overlay and disk-diffusion methods were used to screen for antibacterial activity against pathogenic bacteria MRSA (Methicillin Resistant Staphylococcus aureus) and Staphylococcus epidermidis. Molecular characterization was investigated using PCR amplification 16S rRNA gene sequence. Homology analysis was used by using BLAST to identify the similarity, while phylogenetic tree was constructed by using neighbour joining tree. The result showed that the active strains of bacteria IB.6a.1 (Acc. No. LC002977) belonging to Bacillus subtilis with 95% sequence similarities. These findings suggest that the identified strains may contribute to the search for new sources of antibacterial substances.

Keywords: Bacteria associated, Sargassum, antibacterial activity, 16S rRNA, phylogenetic analysis

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1. Introduction

Seaweeds are one of the large and diverse ecosystems, it plays an essential role in marine environment. It is mainly involved in global primary production and providing food and shelter for variety of organisms. Seaweeds surface supplies protected and nutrient rich conditions for the bacterial growth [1]. Seaweed have a rich diversity of associated microorganisms compare with the other multicellular organisms. These microorganisms maybe beneficial or harmful to the seaweeds. Epiphytic bacterial communities have been reported as vital for morphological development of seaweeds, and bacteria with antibacterial properties are thought to protect the seaweeds from pathogens and the other competition organisms [2]. Some bacterial species show host specificity and bactericidal activity against specific pathogens. This specificity engage complex biochemical interactions between seaweed and bacteria [3].

Brown algae-associated bacteria may degrade algal polysaccharides, such as fucoidan[4], or alginates[5]. Algae can produce biologically active compounds that are capable of killing bacteria or inhibiting bacterial growth[6], [7]. Furthermore, numerous studies have previously reported on antimicrobial compounds of seaweed [8], [9]. Marine bacteria are closely associated with brown algae. Several bacterial species have been isolated from the spot-wounded fronds of Laminaria japonica [10], the surface of Fucus serratus [11], the rotten thallus of Fucus evanescens [12] and Undaria pinnatifida [13]. Moreover, previous investigation bacteria associated with algae having the antibacterial activity [14], [2].

The resistance of bacteria to clinically important antibiotics is a major factor throughout the world. Since methicillin resistance Staphylococcus aureus (MRSA) were first reported in England [15]. MRSA is recently a serious problem because it exhibits multidrug resistant to almost all commercial antibiotics except vancomycin and teicoplanin [16]. While Staphylococcus epidermidis is a natural inhabitant of the normal skin and mucosa. It is also a common cause of septicemia, especially in immunocompromised patients. Infections caused by S. epidermidis are often associated with the use of medical devices [17]. The increasing global resistance of pathogenic bacteria to existing antibiotics has become a public health problem and research efforts are now addressing the discovery of novel and efficient antibacterial compounds [18].

The purpose of this work were to isolate bacteria associated with three species of Sargassum spp. (S. polycystum, S. duplicatum, and S. echinocarphum), to screen antibacterial activity against pathogenic bacteria MRSA (Methicillin Resistant Staphylococcus aureus) and Staphylococcus epidermidis and moreover to investigate characterization molecular by using PCR amplifications 16S rRNA gene sequence.

2. Materials and Methods

2.1. Sampling and isolation of associated bacteria

Three species of brown algae Sargassum spp. (S. polycystum, S. duplicatum, and S. echinocarphum) were collected from Panjang island, Jepara, Central Java, Indonesia by skin diving to a depth approximately 3 m. The isolation of associated bacteria was done at the marine microbiology laboratory at the Marine Station, Diponegoro University, Jepara, Central Java, Indonesia. Sampling site for the collection of associated bacteria of Sargassum spp. from Panjang island (Fig. 1).

During the collection, Sargassum spp. were put into ziplock plastic bags and placed in a cool box. Each Sargassum species was put into a sterile plate and the tissues of each Sargassum were rinsed with sterile seawater and scraped off with a sterile knife. The resultant tissues of each sample were serially diluted, spread on the ZoBell 2216E marine agar medium and incubated at room temperature for 48 h. Based on morphological features, colonies were purified by making streak plates [19].
2.2. Antibacterial test

The screening of antibacterial test was conducted at the Tropical Marine Biotechnology and Natural Product, Central Laboratory For Research and Services, Diponegoro University. Screening of bacterial associated against pathogenic bacteria MRSA and *Staphylococcus epidermidis* was performed by using overlay method. Cultures of each bacterium in the logarithmic phase were mixed with soft agar medium which were poured on to the respective agar surfaces previously inoculated with associated bacteria that had been incubated for 2-3 day at room temperature. Antibacterial activity was defined by clear zones around the associated bacteria [16].

Confirmation of the antibacterial activity was conducted by using agar diffusion method. Associated bacteria was tested against pathogenic bacteria MRSA and *Staphylococcus epidermidis*. One 100 μL culture of tested bacteria in the logarithmic phase were spread on to agar medium. Several paper disks (8mm; Advantec Tokyo Roshi, Ltd, Japan) containing 30 μL of the associated bacteria strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Antibacterial activity was defined by inhibition zones around the paper disk [20]. Isolate that showed antibacterial activity against pathogenic bacteria was chosen for further characterization molecular based on PCR amplifications 16S rRNA gene sequence.

2.3. DNA extraction
DNA extraction was conducted by using chelex method [21]. Selected colonies were inoculated in 50-100 μl ddH2O and 1 ml of 0.5% saponin in PBS 1x (saved overnight). The mixture was centrifuged (12000 RPM, 10 min). Supernatant was discard. Then 100 μl ddH2O and 50 μl of 20% chelex 100 (shake up chelex solution and ensure that some of the crystals make it into sample) were added to a final solution and the solution was boiled for 10 min and vortex once after 5 min. The mixture was sentrifuged (12000 RPM, 10 min) and stored at -20°C. The DNA concentrations were quantified and qualified by using NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of 1 μl DNA sample was determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 260/280 and 260/230 nm ratios was calculated by the NanoDrop spectrophotometer and used to evaluate the DNA purity and also the concentration of DNA.

2.4. PCR amplification 16S rRNA gene sequence

DNA extracts for 16S rRNA genes sequences were amplified by PCR using universal primers 27F (5‘AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5‘TACGGTTAACCTTGTTACGACTT-3′). The PCR mixture consisted GoTaq®Green Master Mix Promega (25 μl), primer 27F (0.5-5 μl), primer 1492R (0.5-5 μl), DNA extract (1-5 μl), and Nuclease-Free Water (50 μl).

The PCR reaction was performed in a MJ Mini Personal Thermal Cycler (BIO RAD) using cycling conditions consisting of an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 oC for 1 min, and extension at 72 oC for 1 min. A final extension was performed at 72 oC for 7 min [13]. The PCR products were analyzed by agarose 1 % gel electrophoresis and the result showed by using UVIDoc HD5 (UVITEC cambridge).

2.5. DNA sequencing

DNA sequencing was conducted at Macrogen Laboratory, Korea. Purification PCR product was used QIAquick PCR purification KIT (QIAGEN) and for PCR sequencing was performed by using Big Dye Terminator v.3.1 and automatically sequences analysis by using ABI 3130XL, Applied Biosystem. The sequences of amplified 16S rRNA genes were deposited in the GenBank database the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The sequences were inserted to the Advanced BLAST search program to identify the sequences of any closely related organisms [22]. The partial 16S rRNA sequence of the strain associated bacteria was deposited in DDBJ (DNA Data Bank of Japan).

2.6. Phylogenetic Analysis

The result of DNA sequences were preliminarily aligned with ClustalW Multiple Alignment and the phylogenetic analyses were performed by using MEGA 6. The phylogenetic trees were determined using the neighbour-joining method with Kimura's two-parameter. The resultant tree topology was evaluated by bootstrap analyses of the neighbour-joining method based on 1.000 resamplings [16].

3. Results

3.1. Isolation of associated bacteria

The total associated bacteria of each of species from Sargassum spp. were twenty-three strains marine bacteria associated. They were 6 strains bacteria from S. polycystum, 9 strains bacteria from S. duplicatum, and 8 strains bacteria from S. echinocarphum.
3.2. Antibacterial activity

Screening of antibacterial activity of associated bacteria of the *Sargassum* spp. against pathogenic bacteria with overlay method, resulted in a total of 3 active isolates that inhibited both of pathogenic bacteria from 23 strain associated bacteria. Confirmation for choosing the best strain bacterium was performed by using disk-diffusion method and the result showed that isolate IB.6a.1 inhibited the growth of both of pathogenic bacteria (Table 1).

| Code    | MRSA (mm) | *S. epidermidis* (mm) |
|---------|-----------|-----------------------|
|         | replication 1 | replication 2      | replication 1 | replication 2      |
| IIIB.6b.1 | -          | -                    | 3,7±0,5       | 4,2±0,5            |
| IB.6a.1  | 3,6±0,4    | 3,9±0,5              | 5,9±0,6       | 4,7±0,4            |
| IIIB.8a.2 | -          | -                    | 3,7±0,4       | 4,5±0,5            |

3.3. Molecular characterization and Phylogenetic analysis

The result from BLAST homology of bacterial strain IB.6a.1 showed that this bacterium is affiliated to the genus *Bacillus*. The isolate IB.6a.1 come from *Sargassum polycystum*. The phylogenetic tree showed that isolate IB.6a.1 is most closely related with *Bacillus subtilis* with 95 % sequence similrities (Fig. 2). The partial 16S rRNA sequence of this strain active associated bacteria was deposited in DDBJ (DNA Data Bank of Japan) with accession number LC002977.

![Fig 2](image-url). Neighbor-joining phylogenetic tree based on comparative 16S rRNA gene sequence analysis of *Bacillus subtilis* showing the phylogenetic affiliation of strain IB.6a.1. Selected sequences *Deinococcus radiodurans* was used as an outer group.
4. Discussion

In the present work, we have isolated 23 strain marine bacteria associated with three species of brown algae Sargassum spp. (S. polycystum, S. duplicatum, and S. echinocarpum). Based on previous research, bacteria associated had been isolated from green algae Halimeda sp. from Lake Kakaban, Indonesia, they were 7 strain bacteria associated [23]. On the other hand, there were 66 strain bacteria associated that isolated from brown algae Undaria pinnatifida [13]. These bacteria could acquire the necessary nutrition such as vitamin, polysaccharide an fatty acid from their animal or plant hosts; while on the other, they could excrete products such as amino acid, antibiotic and toxin propitious for the development and metabolism of the hosts, or to improve the chemical defense capability of the hosts [24].

Inhibition zone among associated bacteria from Sargassum spp. are the great interest to search for antibacterial substances. Isolation and screening for secondary metabolite-producing bacteria have been strongly investigated. Our results showed that one strain associated bacteria from Sargassum IB.6a.1 have inhibition zone against MRSA 3.6±0.4 and 3.9±0.5 mm while against S. epidermidis 5.9±0.6 and 4.7±0.4 mm. According to the previously research showed that bacteria associated from brown algae Sargassum spp. had antibacterial activity with the inhibition zone between 0.5-4.5 mm against pathogenic bacteria Staphylococcus aureus, Staphylococcus epidermidis, Serratia marcescens. Salmonella thyphi, Klebsiella pneumoniae, and Salmonella enteritidis [25]. Moreover, bacteria associated from Gracilaria corticata had antibacterial activities with inhibition zone 8.5 mm; Padina gymnospora 5.8 mm and Valoniopsis pachynema 13.8 mm against pathogenic bacteria Staphylococcus sp [2].

The resulted BLAST homology from NCBI (National Center for Biotechnology Information) showed that the active strain bacteria IB.6a.1 95 % identical to Bacillus subtilis base on 16S rRNA gene sequence. These strain has been submitted to DDBJ (DNA Data Bank of Japan) with the accession number LC002977. Based on the previous research showed that bacteria associated from brown algae Undaria pinnatifida have similarities with Psychrobacter aquimaris, P. celer, P. nivimaris, P. pulmonis, Psychromonas arctica and Bacillus psychrodurans base on 16S rRNA gene sequence [14]. Bacteria associated with algae Gracilaria corticata have similarities with Pseudoalteromonas sp., and Pseudomonas sp.; from algae Padina gymnospora have similarities with Alteromonas sp; and from algae Valoniopsis pachynema have similarities with Oceanobacillus sp. and Bacillus sp [2].

Some bacteria were isolated with high antibacterial activity against fouling bacteria belonged to Bacillus sp. [26]. Bacteria associated from genera Bacillus, Pseudomonas, Pseudoalteromonas, and Paracoccus that has been isolated from algae have antibacterial activity [14]. Bacillus subtilis is the best associated bacteria that can inhibit pathogenic bacteria. The genus Bacillus recognised as prolific producers of antibiotics [27], [28], it’s species produce biologically active lipopeptides [29], anti-HIV activity [30]. The isolat of Bacillus pumilus associated with the marine sponge Ircinia sp. produces surfactin-like substances, cyclic acyldepsipeptides, called bacircines 2, 3, 4, 5 and 5A.

5. Conclusion

Bacteria associated from brown algae Sargassum spp. represents potential sources of antibacterials against MRSA and Staphylococcus epidermidis. The active strain bacteria associated IB.6a.1 (Acc. No. LC002977) belonging to Bacillus subtilis with 95 % sequence similarities. The member of the genus Bacillus shows significant antibacterial activity against the tested bacteria.

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