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Molecular Identification of Symbiont Bacteria on Brown Alga *Padina australis* Hauck

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Abstract: The research aimed to identify the symbiont bacteria in brown alga *Padina australis* from the coastal waters of Molas, Manado city, Indonesia. Fresh samples were crushed with a mortar and pestle until smooth. A total of 1 g of the smooth alga was added into a test tube containing 9 mL of distilled water to obtain a 10-1 dilution. Next, 1 mL of a 10-1 solution was put into another test tube containing 9 mL of distilled water and vortexed for 15 minutes to obtain 10-2 dilution and so on until a 10-8 solution was obtained. 100 µL of solution from each series 10-4 – 10-8 was spread on NA media and incubated at a temperature of 27–29°C for 24 hours. DNA extraction was carried out following the modified Genomic DNA Mini Kit (Geneaid) protocol. DNA amplification was carried out using the 16S rRNA gene. Amplification using primers for the 16S rRNA gene BKXF (forward) and BKXR (reverse) resulted in fragments 1152 base pairs in length. The results of BLAST analysis of SK1 symbiont bacterial isolates found a Max score and total score of 1369, a query coverage of 97%, and a Percent Ident of 97.73%, which showed similarity to bacterial nucleotides of Bacillus cereus.

Keywords: 16S rRNA gene; BLAST analysis; DNA extraction; Symbiont bacteria

Introduction

Research on brown algal symbiont *Padina australis* from the coastal waters of Molas, Manado City, Indonesia, involved a detailed process of sample collection, dilution, and bacterial isolation. The study aimed to identify the dominant bacterial symbiont using molecular techniques, specifically focusing on the 16S rRNA gene for DNA amplification. Findings revealed a bacterial isolate, SK1, with significant genetic similarity to known bacterial sequences, as shown by BLAST analysis results. This study contributes to the understanding of microbial communities associated with brown algae and their potential role in marine ecosystems. Indonesia is very rich in marine natural resources, one of which is marine algae. Various species of marine algae are found in Indonesian marine waters. With the development of technology and the increasing needs of society, algae that have economic and ecological value have begun to be explored to find out their various benefits. Algae, especially macroalgae, have the potential to be used as a promising source of bioactive compounds in the medical world (Leary et al., 2009). Brown algae have been identified to contain pigments (fucoxanthin, astaxanthin, carotenoids) and polyphenols (phenolic acids, flavonoids, tannins) that function as antioxidants, antimutagenics, anti-coagulants, anti-tumors, and regulators of lipid metabolism. The secondary metabolites present in brown algae consist of alkaloids, glycosides, tannins, and steroids, which are useful for medicine and the pharmaceutical industry.

Brown algae host diverse bacterial communities that play important roles in their biology and ecology. These bacteria are involved in processes such as polysaccharide degradation and nutrient cycling, which are critical to the health of marine ecosystems (Lin et al., 2016). Bacteria are microorganisms that live in various

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types of habitats, including water. These microorganisms have a very important role as constituents of ecosystems because they act as degraders. In addition, bacteria are potential sources of bioactive compounds (Nofiani, 2012). The ability of bacteria to produce bioactive compounds may be associated with the way the bacteria live as symbionts with marine organisms, one of which is brown algae. Brown algae themselves have been reported to produce various metabolite compounds that function as anti-obesity, antidiabetic, anticancer, antioxidant, and antibacterial agents as well (Lin et al., 2016; Nurrahman et al., 2018).

Symbiotic bacteria from brown algae, including *P. australis*, have shown antibacterial activity against various pathogens, indicating their potential for biotechnological applications. Continuous exploitation and utilization of metabolite compounds produced by algae may cause the extinction of algae species. Thus, the use of symbiont bacteria on algae as a source of bioactive materials is one of the best solutions to prevent such extinction. The reasons behind this are that bacteria are easy to grow, have a short life cycle, and can produce large amounts of bioactive compounds in a short time (Nurzakiyah, 2016). Utilizing bacteria reduces pressure on algae populations, thus contributing to the conservation of marine ecosystems. This approach aligns with sustainable development goals by promoting the use of renewable biological resources (Nemtseva et al., 2018). The identification of bacteria is the first step in efforts to utilize metabolite compounds produced by symbiotic bacteria. Research related to symbiotic bacteria has been widely carried out, including on the brown algae *Padina sp*. (Kandio et al., 2021), the red algae *Galaxaura regosa* (Hamzah et al., 2018), and the green algae *Halimeda opuntia* (Rau et al., 2018).

Based on observations in the field, the brown algae *Padina australis* are often found abundantly in various water areas in North Sulawesi, Indonesia. This research aimed to identify the symbiont bacteria on brown alga *P. australis* from the coastal waters of Molas, Manado, North Sulawesi Utara. The novelty of this research is the use of the symbiont bacteria from *Padina australis* for medicine and the pharmaceutical industry.

Method

Samples Collection

Samples of P. australis were collected from the coastal waters of Molas Village (Figure 1). Samples were gathered by the purposive sampling method during the lowest tide where the depth of water was about 20 – 30 cm. Live algae were removed from the substrate and immediately cleaned of impurities using seawater. The total number of samples taken was 5 specimens. The collected samples were put in zip-lock plastic bags, placed in a cool box, and then transported to the Laboratory of Biology, Faculty of Mathematics and Natural Sciences (MIPA UNSRAT), Sam Ratulangi University, Manado, for analysis.

Figure 1. Map of research location

Isolation of Symbiont Bacteria

Isolation of symbiont bacteria in brown algae *P. australis* was carried out using the spread method (Madigan et al., 2005). Fresh samples of brown alga were crushed with a mortar and pestle until smooth. Afterward, a total of 1 g of the smooth alga obtained was added into a test tube containing 9 mL of distilled water to obtain a 10^{-1} dilution. Next, 1 mL of the 10-1 solution was taken and put into another test tube containing 9 mL of distilled water and vortexed for 15 minutes to obtain a 10-2 dilution. The same procedure was carried out until a 10-8 solution was obtained. To identifying symbiotic bacteria, 100 µL of solution from each series 10-4 – 10-8 was taken and spread on NA media in a Petri dish and incubated at a temperature of 27–29°C for 24 hours.

The symbiont bacterial colonies that grown were observed for their morphology, including shape, color, edges, and elevation. Using an inoculating loop, different macroscopic characteristics of the bacterial colonies were reinoculated by the quadrant method on solid NA medium, then incubated at a temperature of 27–29°C for 24 hours. After the bacteria grew and a pure culture was obtained, the bacterial isolate was purified again onto slanted agar medium and onto a Petri dish using the scratch method and incubated for 24 hours. The results of the pure culture were then used for the identification process.

Molecular Identification of Symbiont Bacteria
DNA Extraction

DNA extraction of the most dominant bacterial cultures was carried out following the modified

Genomic DNA Mini Kit (Geneaid) protocol. DNA amplification was carried out using the 16s rRNA gene. The 16S rRNA gene is most often used in both eukaryotes and prokaryotes. The PCR product was then analyzed using electrophoresis on an agarose gel with a concentration of 0.8%. The electrophoresis process was carried out for 40 minutes. Visualization was carried out using certain dyes and detected with UV light on a UV-trans illuminator (Umar, 2015). The results of this detection were documented using a special tool called the gel documentation system (gel-doc).

Amplification of the 16S rRNA Gene by PCR

The PCR technique was carried out using the MyTaq HS Red Mix Kit (Bioline), 16S rRNA forward (BKXF) and reverse (BKXR) primers, and sterile Milli-Q water. The PCR kit contains $MgCl_2$ and a reaction buffer. The 16S rRNA gene is most often used in both eukaryotes and prokaryotes.

Electrophoresis

Electrophoresis was carried out using a 0.8% agarose gel that had been flooded with TBE 1X. The gel was soaked in a mixture of Tris-Borate-EDTA and ethidium bromide solutions. The results of electrophoresis were observed by placing the agarose gel in a UV-transilluminator and the results were documented using a camera.

Sequencing

The PCR product obtained and both primers were sent to First Base CO (Malaysia) for sequencing purposes. The results obtained were presented in a chromatogram showing the DNA sequence resulting.

Result and Discussion

Symbiont Bacteria

Seven isolates of symbiotic bacteria on P. *australis* were found in this research, symbolized as SK1, SK2, SK3, SK4, SK5, SK6, dan SK7. These isolates were purified to get pure colonies and then their macroscopic and microscopic characteristics were observed. Purification is the process of purifying or separating bacterial colonies so that only pure bacteria are obtained (Pamaya et al., 2018). Macroscopic characteristics included shape, color, surface, and edges of the colony, while microscopic characteristics were observed through Gram Stainning (Tables 1 and 2).

Table 1. Morphological Characteristic of Symbiont Bacterial Colonies on *P. australis*

Isolates	Colony Morphology			
	Shape	Color	Surface	edge
SK 1	round	White	flat	smooth
SK 2	round	White bone	flat	choppy
SK 3	round	White bone	Raised	smooth
SK 4	Rhizoid	white	flat	choppy
SK 5	round	yellowish	flat	smooth
SK 6	round	White bone	flat	irregular
SK 7	round	white	flat	choppy

Table 2. Microscopic Characteristics of Symbiont Bacterial Colonies on *P. australis*

Isolates	Characteristic
SK 1	basil
SK 2	coccus
SK 3	basil
SK 4	coccus
SK 5	coccus
SK 6	coccus
SK 7	coccus

Identification of Symbiont Bacteria by 16s rRNA Gene

Molecular identification was carried out on the most dominant isolate, namely SK1. The result obtained from First Base Co., Malaysia, was then analyzed in GenBank using BLAST analysis. The electrophoresis results of isolate SK1 showed that there was a genomic DNA clearly visible band. Amplification using primers for the 16S rRNA gene BKXF (forward) and BKXR (reverse) found a fragment 1152 base pairs in length (Figure 2). This was in line with the DNA ladder used, namely a 1 kb DNA ladder used as a marker, and which can measure DNA lengths of 250–10,000 bp. In this amplification process, DNA was multiplied in certain areas limited by the primers. These PCR results were then used for the DNA sequencing stage (Rahayu et al., 2015). DNA Extraction and Amplification: DNA of isolate SK1 was extracted and amplified using primers specific for the 16S rRNA gene, yielding a base pair product of 1152. This is consistent with the use of a 1kb DNA ladder for size estimation. The amplified DNA was sequenced, and the sequences were analyzed using BLAST to identify the closest known bacterial species. This method is effective for species-level identification, as shown in a similar study in which 16S rRNA sequencing was used to identify bacterial strains with high accuracy.

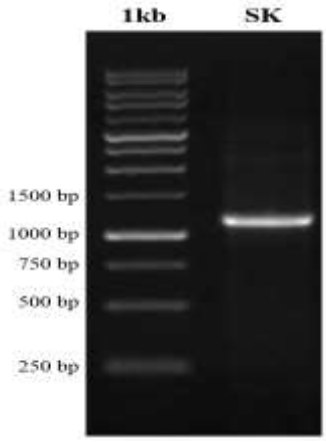

Figure 2. Visualization of 16S rRNA gene amplification of isolate SK1 on 0.8% agarose gel concentration

The result of 16s rRNA gene sequence of isolate SK1 was displayed in a chromatogram which was then edited to correct any overlaps. Editing was carried out by cutting the forward and reverse primers, which were the starting points for DNA replication. This follows Kolondam (2015) statement that the amplification primers must be edited. The nucleotide base sequence of the edited chromatogram was obtained in FASTA (Fast Alignment) form (Monalisa et al., 2019), followed by sequence matching in GenBank using BLAST. Primer editing is an important step in the 16S rRNA sequencing workflow. It involves removing primers from sequence data to prevent any interference with subsequent analysis. This step is crucial for obtaining accurate sequence data that can be used for reliable taxonomic identification. The 16S rRNA gene is a widely used phylogenetic marker due to its presence in all bacteria and the presence of conserved and variable regions that allow differentiation between species. The process of sequence matching and analysis is enhanced using next-generation sequencing technology, which enables high-throughput and accurate identification of microbial communities. The use of proofreading polymerase, which has 3' to 5' exonuclease activity, allows correction of misincorporated bases and editing of PCR primers to match the template sequence. This increases the accuracy of the sequencing process (Gohl et al., 2021; Naqib et al., 2018).

DNA sequences that had been converted into FASTA format were entered as a query in GenBank NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov) to obtain information about the types of microorganisms that have the highest similarity level to microbial samples. The FASTA format is a text-based file format used to represent nucleotide sequences or amino acid sequences encoded in single-letter characters (Daryl et al., 2021).

Files in the FASTA format can represent a single sequence or multiple sequences in one file (Haryanto, 2010). The results of BLAST analysis of SK1 symbiont bacterial isolates from P. australis found a Max score and total score of 1369, with the query coverage closest to 100% (97%), and a Percent Ident of 97.73% (Table 3).

Table 3. BLAST Analysis of Symbiont Bacteria (Isolate SK1) on *P. Australis*

Description	Scientific Name	Max Score	Total Score	Query Cover	Per. Ident	Acc. Len	Accession
Bacillus cereus strain SBMAX25	*Bacillus cereus*	1369	1369	97%	97.73%	1422	MK796028.1
Bacillus sp. (in: firmicutes) strain UFLA WFC552	*Bacillus sp.*	1369	1369	97%	97.73%	1392	KY660409.1
Bacillus cereus strain GSI6	*Bacillus cereus*	1369	1369	97%	97.73%	1412	MG711835.1
Bacillus cereus strain HD1	*Bacillus cereus*	1369	1369	97%	97.73%	1432	KY773580.1
Bacillus sp. (in: firmicutes) strain G29	*Bacillus sp.*	1369	1369	97%	97.73%	1420	KX343988.1
Bacillus paramycoides strain HBUAS67266	*Bacillus paramycoides*	1369	1369	97%	97.73%	1473	OQ804846.1
Bacillus sp. (in: firmicutes) strain B6	*Bacillus sp.*	1369	1369	97%	97.73%	1425	OQ726341.1
Uncultured Bacillus sp. clone C4A04	uncultured *Bacillus sp.*	1369	1369	98%	97.49%	1460	KP016667.1
Uncultured bacterium clone 11_St_11	uncultured bacterium	1369	1369	97%	97.73%	1493	KM464090.1
Bacillus thuringiensis strain HBUAS58106	*Bacillus thuringiensis*	1369	1369	97%	97.73%	1474	ON209899.1

Molecular analysis showed that the isolate SK1 from P. australis had a length of 1152 bp. BLAST analysis found a Max score and total score of 1369, a query coverage of 97%, and a Percent Ident of 97.73%, which showed similarity to bacterial nucleotides of B. cereus. The finding of this research was like research conducted by Sihombing (2018) who successfully identified B. *cereus* and *B. thuringiensis* molecularly on *Padina sp*. with the percentage of nucleotide length compared to the nucleotide sequence in the BLAST being 100% and 16S rRNA sequence similarity of more than 97%. 16S rRNA sequence similarity between 93%–97% can represent identity at the genus level but difference at the species level, whereas if it is below 93%, it is possibly a new

species whose nucleotide base sequence has not been listed in the gene bank database (Copper, 2010).

Molecular analysis of isolate SK1 from *P. australis*, which showed 97.73% similarity to *Bacillus cereus*, is in line with the findings of previous studies that identified *B. cereus* and *B. thuringiensis* in the same context. This high percentage similarity indicates a close genetic relationship, usually indicating the same genus but potentially different species, as 16S rRNA sequence similarity between 93% and 97% may represent identity at the genus level but not necessarily at the species level. This finding is consistent with research (Sihombing, 2018), which also identified *B. cereus* and *B. thuringiensis* with high nucleotide sequence similarity. Virulence genes: *B. cereus* is known for its virulence factors, which include enterotoxin genes such as NHEA, CytK, and entFM. These genes contribute to its pathogenicity and are commonly found in foodborne strains (Mostafa et al., 2024). Whole-genome sequencing of *B. cereus* strains revealed a complex genetic structure with many plasmids and a large number of coding sequences, which contribute to its adaptability and pathogenic potential (Li et al., 2024; Premkrishnan et al., 2021).

Isolate SK1 was similar to *B. cereus* strain HBUAS67266 with a homology level of 97.73%. This means that the level of homology is the same at the genus level but different at the species level. *B. cereus* has macroscopic characteristics of round colonies, flat surfaces, and flat edges, while the microscopic characteristics are rod-shaped cells and Gram-positive (Hidayat et al., 2014). The isolate SK1 has similar characteristics, namely round colonies, flat surfaces, flat edges, rod-shaped cells, and Gram-positive. Similar research also successfully identified molecularly the symbiont bacteria on brown algae *Padina sp*., namely B. *cereus*. It was found that B. *cereus* also lives in symbiosis with the sponge *Clathrina sp*. and *Agelas sp*. (Hidayat et al., 2014).

B. cereus is a mild, harmless saprophyte found in soil, water, air, and plants. This bacterium is capable of forming endospores that are resistant to high temperatures (Awetz et al., 2013). *B. cereus* is strongly proteolytic because it produces enzymes (protease, amylase, lecithinase, etc.) that can break down protein and has almost the same properties as renin, so it can coagulate milk (Fardiaz, 1998). This bacterium also ferments carbohydrates (glucose and mannose). In addition, it will grow at a pH of 4.3-9.3 and a water activity (Aw) of 0.95 (Feliatra et al., 2018).

B. cereus is a probiotic bacterium that is able to inhibit the growth of pathogenic bacteria such as Vibrio sp. (Feliatra et al., 2018). This bacterium produces secondary metabolites or bioactive compounds that suppress the growth of other bacteria (El-Sharouny et al., 2015). *B. cereus* has the potential to act as an antibacterial against the *Multi Drug Resistance Organism* (MDRO) *Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa* ESBL, or *Stapyhlococcus lugdunensis* MRSA. This heterotrophic bacterium can also degrade toxic organic materials in the environment, especially in aquatic environments.

Conclusion

Molecular analysis using the 16S rRNA gene on the most dominant symbiont bacterium (isolate SK1) in P. australis showed 97.73% similarity to the bacterial species B. cereus. This symbiont bacterium has macroscopic characteristics of round colonies, flat surfaces, and flat edges, while the microscopic characteristics of this bacterium are rod-shaped cells and Gram-positive. The practical implication of this research is the use of the symbiont bacteria from Padina australis for medicine and the pharmaceutical industry.

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Author Contributions

MFOS, SU and DK were involved in concepting and planning the research. MFOS, SK, dan AMT performed the data acquisition. MFOS, SK and BK analyzed the experimental data, MFOS drafted the manuscript. Each author contributed to the manuscript's critical editing.

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Conflicts of Interest

The authors declare no conflict of interest.

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