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NIKEN DHARMAYANTI♥, ARMA ANTI, RESMI RUMENTA SIREGAR, YULIATI H. SIPAHUTAR, AEF PERMADI, ARPAN NASRI SIREGAR, RANDI BOKHI SALAMPESSY, SUJULIYANI, SITI ZACHRO NURBANI, HENI BUDI PURNAMASARI

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Abstract. *Dharmayanti N, Anti A, Siregar RR, Sipahutar Y, Permadi A, Siregar AN, Salampessy RB, Sujuliyanti, Nurbani SZ, Purnamasari HB. 2021. Title. Biodiversitas 22: 373-377.* Brown seaweeds have the potential to produce bioactive compounds. Bacteria associated with seaweeds are involved in the production of metabolites. Microbes may be present as a living symbiotic in association with other algae as epiphytes or endophytes. In this study, bacteria isolated from brown seaweed (*Turbinaria conoides*) were tested for antibacterial activity. A total of 14 bacteria were isolated, of which 6 were isolated from external tissue, while 8 from internal tissue. Results of an antagonistic test revealed that 7 isolates showed inhibitory activity against *Staphylococcus aureus* and only 1 isolate showed the inhibition against both *S. aureus* and *Escherichia coli*. Phenotypic and genotypic analysis showed that the symbiont bacteria was *Lactobacillus plantarum.*

Keywords: Bioassay, brown seaweed, antagonistic, diffusion paper disc, *Lactobacillus plantarum*

INTRODUCTION

Seaweed is one of the largest producers of biomass from the ocean and is a potential source of new, diverse, and unique compounds (Bahare et al. 2019). Many substances are obtained from seaweed, such as alginates, carrageenan, and agar, which have been used for decades in traditional medicine, pharmacology, and food (Andrea et al. 2019). Other compounds have bacteriostatic or antibacterial, antiviral, anti-tumor, anti-inflammatory, and antifouling activity. Therefore, seaweed can provide promising bioactive that can be used in the treatment of human diseases or new antimicrobial agents to replace synthetic antibacterial agents used in agriculture and the food industry. Seaweed applications are particularly used in the design of new antimicrobial drugs. Research for the identification of promising algal species, standardization of analytical methods, isolation of compounds through integrated fractionation of bioassays, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects between components, and efforts to improve yields and lowering extraction costs is needed (Marie et al. 2016).

In later decades, strides microbiological procedures have altogether made a difference in build-up phylogenetic affiliations of seaweed-related epi-bacterial communities and endophytes. Be that as it may, there is inadequately prove that utilitarian connections for seaweed-bacterial intuitive can be built up and well caught on. Epiphytic bacterial communities are rapid colonizers of the ocean growth surface, some of the time versatile and able to quickly metabolize algal exudates (Singh and Reddy 2014). It has traditionally been used for children's fever, as a fertilizer, repellent, including antioxidant, anti-inflammatory, antimicrobial, and anti-cancer (Gupta and Abu-Ghannam 2011). Seaweeds can secrete secondary metabolites with antibacterial properties (Shannon and Abu-Ghannam 2016). The symbiotic mutualism occurs as algae provide essential sites and nutrients, while the bacteria encourage growth and protect the algal surface against symbiont bacteria isolates as algae have abundant antimicrobial activity. The existence of the bacteria is suspected as a result of infections acquired from the community (Arumugama et al. 2017). *Turbinaria conoides* is a tropical marine alga widely distributed in coastal waters in Asia.

This study evaluates the properties of the brown alga *Turbinaria conoides* in producing bioactive compounds including the inhibition of human pathogens.

MATERIALS AND METHODS

Sampling

Samples of *Turbinaria conoides* (about 1 kg wet weight) were taken from Lima island, Serang City, Banten, Indonesia (S: -6.001051; E: 106.153804). Samples were maintained in fresh seawater for laboratory analysis within 24 hours of collection.

Isolation of symbiont bacteria producing antibacterial compounds

Bacteria were isolated in a solid medium and the size of the colony was different for each species and was characteristic of a particular species (Sanders 2012).

Epibionts were extracted from 15 grams of algae by rinse in with 30 mL of sterile seawater. The rinse water was incubated in 30 mL of nutrient broth medium and shaken at room temperature for 24 hours. The bioactive compound was extracted by crushing 15 g of alga with a mortar and pestle with the addition of 15 mL of sterile seawater. The suspension was insert into a 30 mL nutrient broth medium and shaken at room temperature for 24 hours.

After the extraction process, 1 mL of refresh samples were diluted in a 9 mL of sterile nutrient broth to make 10^{-1} dilution. This process was continued to achieve 10^{-5} dilutionEach dilution was grown on a plate count agar medium by incubating them at 37°C for 2 x 24 hours. Colonies of bacteria that produce antimicrobial compounds were characterized by a clear zone. Furthermore, the colonies with stable inhibition zones were collected by isolating them on a slant agar medium.

Selection of symbiont bacteria isolates antagonistically against pathogenic bacteria

For this, a qualitative test was carried out directly by scratching the isolates on the surface of the media that has been dispersed with two test bacteria, i.e. *Escherichia coli* and *Staphylococcus aureus* (Monte et al. 2014)). The media was then incubated for 48 hours at 37°C. Each scratching round of isolates was then marked by a unique code. Inhibition zones were showing clear zones around the colony of symbiont bacteria isolates for both *E. coli* and *S. aureus*. Strains that showed maximum antagonistic effect against tested pathogens were identified. Strains showing maximum antagonistic effects were isolated and selected for antibacterial testing by the paper disc diffusion method. Further, the strains were identified at the phenotypic and genotypic levels.

Antibacterial potential testing of symbiont bacterial isolate by paper disc diffusion

Antibacterial testing of symbiont bacteria for inhibitory growth of *E. coli* and *S. aureus* was performed by the paper disc diffusion method (Grela et al. 2018). The supernatant was obtained by separating the filtrate and the supernatant was centrifuged for 1 hour (25°C and 3000 rpm). Paper discs containing 40 µL supernatant was considered as the treatment while 40 µL nutrient broth was used in negative control and chloramphenicol (0.01 mg/mL) was used as a positive control. After that, the discs were placed on the surface of the Mueller Hinton Agar medium containing 1 mL test bacteria and incubated for 48 hours at 37°C. The supernatant diffuses from the disc into the agar. The presence of a clear zone around the supernatant and antibiotic discs was measured by the meter rule in mm. The zone sizes were compared to assess bioactivity as sensitive, resistant, or intermediate, and the presence of a clear zone was measured by the meter rule in mm.

Identification of symbiont bacteria phenotype and genotype

General bacterial identification based on colony characteristic observations on liquid medium and solid medium followed by cell morphology (gram staining, spore staining, and Ziehl-Neelsen staining), and biochemical test (motility, gelatin hydrolysis, citrate, urease, carbohydrates, and catalase) as described by Phumudzo et al. (2013). The initial selection of isolates from mixed cultures was carried out after enrichment and planting of *T. conoides* samples on the agar medium. The plates were incubated at 37°C temperature for 24 to 48 hours. The data obtained from the bacterial isolate characterization were used to estimate the type of symbiotic bacteria isolated from *T. conoides*. The DNA of the symbiont bacteria isolated was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. The PCR reaction used a PCR machine (Eppendorf German) with a first pre-denaturation at 94 ° C for 90 seconds, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, primary attachment at 50°C for 30 seconds, and extension at 72°C for 90 seconds, followed by the elongation phase at 72°C for 5 min and cooling at 4°C for 20 min. Molecular identification was done through partial genetic analysis of 16S rDNA. DNA extraction was performed using the GES method (Pitcher et al. 1989 modified). PCR Amplification on 16S rDNA using Primer 9 F: 5`- AAG GAG GTG ATC CAG CC-3` and Primer 1541 R: 5` - GAG TTT GAT CCT GGC TCA G - 3` (White et al. 1990, O`Donnell 1993). The analysis of nitrogen base sequence readings was performed with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The next sequenced raw data were trimmed and assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequencing data were assembled in BLAST with genomic data registered in DDBJ/DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/).

RESULTS AND DISCUSSION

The result of symbiont bacteria isolation

A total of 14 colonies were isolated, of which 6 were from epibionts while the other 8 were from algal tissue. The macroscopic results of colonies on mixed cultures can be seen in Table 1, and on slant agar can be seen in Table 2.

The selection results symbiont bacteria producing antibacterial compounds

Based on the results of the direct challenge test, only 5 bacterial isolates i.e. TUL2-B1-2, TUL2-B2-2, TUD2-D2-2, TUD2-D3-2, and TUD3-F-2 showed inhibitory activity against *S.aureus* whereas only 2 viz. TUD4-C1-2 and TUD4-C2-2 bacterial isolates showed inhibition zones against both pathogenic bacteria. The inhibition activity was found to be lower in *E. coli* than in *S. aureus* (Figures 1 and 2).

Isolates with code TUD4-C2-2 had the best inhibition zone. Bacterial isolates derived from algal tissue showed better inhibition than isolates derived from epibionts. The inhibitory zone and diameter measurement results against *S. aureus* and *E. coli* can be seen in Figure 3 and Table 3.

Positive controls showed 16.8 mm inhibition zone against *S. aureus* and 13 8 mm inhibition zone against *E. coli*. Chloramphenicol with a concentration of 0.03 mg on a

paper disc is highly active if its inhibition zone is more than 18 mm (Mounyr et al. 2016), while the dose of chloramphenicol (positive control) used was less than 0.01 mg, so it can be said that bacteria test was found to be sensitive to the positive control. Negative control (NB without symbiotic bacterial inoculation) indicates the absence of activity or inhibition zone, so it can be ascertained that supernatant does not affect the activity formed.

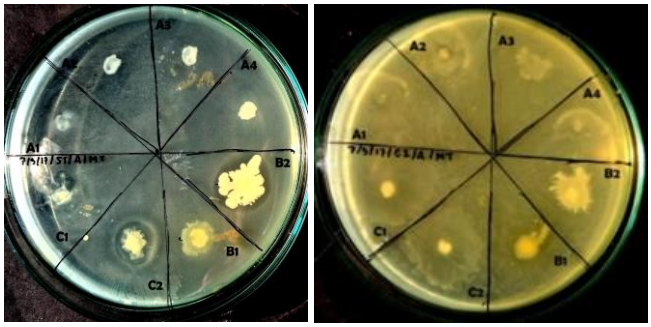
Tabel 1. Macroscopic forms of bacterial colonies.

Colony code	Morphology of colonies			
	Shape	Color	Edges	Elevation
TUL[2]-A1-2	Round	White	Flat	Convex shiny
TUL[2]-A2-2	Round	White	Flat	Convex shiny
TUL[2]-A3-2	Round	White	Flat	Convex shiny
TUL[2]-A4-2	Round	White	Flat	Convex shiny
TUL[2]-B1-2	Round	White	Crooked	Convex shiny
TUL[2]-B2-2	Round	White	Crooked	Convex shiny
TUD[4]-C1-2	Round	White	Flat	Convex shiny
TUD[4]-C2-2	Round	White	Flat	Convex shiny
TUD[2]-D1-2	Round	White	Crooked	Convex shiny
TUD[2]-D2-2	Round	White	Crooked	Convex shiny
TUD[2]-D3-2	Round	White	Crooked	Convex shiny
TUD[2]-D4-2	Round	White	Crooked	Convex shiny
TUD[5]-E-2	Round	White	Flat	Convex shiny
TUD[3]-F-2	Round	White	Flat	Convex shiny

Note: *The code of isolates TUL/TUD states the isolates originating from the outer/inner algae. ** The code of isolates ([2]), ([4]), ([5]), ([3]) states isolates obtained from the dilution. *** The code of isolates A1 and so on, B1 and C1 so on, D1 so on, E, F, the letter codes denote the observed gradient sequence and the number code representing isolates in the order of the first, second, and so on according the best inhibition zone of each colony observed on the plate. **** The code of number 2 identifies the isolate obtained from the second repeat

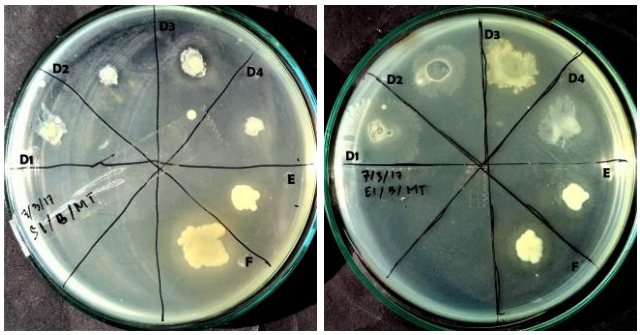
Table 2. Macroscopic form of the isolates on slant agar

Code of isolates	Solid medium	
	Shape	Color
TUL[2]-A1-2	Spread	Milky white
TUL[2]-A2-2	Spread	Milky white
TUL[2]-A3-2	Spread	Milky white
TUL[2]-A4-2	Spread	Milky white
TUL[2]-B1-2	Rhizoidal	Cloudy white
TUL[2]-B2-2	Rhizoidal	Cloudy white
TUD[4]-C1-2	Spread	Milky white
TUD[4]-C2-2	Spread	Milky white
TUD[2]-D1-2	Rhizoidal	Cloudy white
TUD[2]-D2-2	Rhizoidal	Cloudy white
TUD[2]-D3-2	Rhizoidal	Cloudy white
TUD[2]-D4-2	Rhizoidal	Cloudy white
TUD[5]-E-2	Spread	Milky white
TUD[3]-F-2	Spread	Milky white



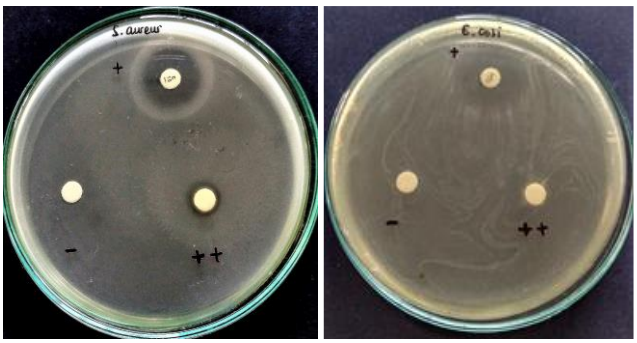
A B

Figure 1. Symbiont bacterial isolates (A1, A2, A3, A4, B1, B2, C1, C2) on a direct challenge test to *Staphylococcus aureus* (A) and *Escherichia coli* (B)



A B

Figure 2. Symbiont bacterial isolates (D1, D2, D3, D4, E, F) on a direct challenge test to *Staphylococcus aureus* (A) and *Escherichia coli* (B)



A B

Figure 3. Results of antibiotic susceptibility test against: A. *Staphylococcus aureus* and B. *Escherichia coli*

Table 3. Results of inhibitory zone diameter

Repetition	The diameter of zone inhibition (mm)					
	Gram-positive			Gram-negative		
	Symbiont bacterial (++)	Control (+)	Control (-)	Symbiont bacterial (++)	Control (+)	Control (-)
1	5.5	16	0	0	13.5	0
2	7.8	17.5	0	0	14	0
Average	6.7	16.8	0	0	13.8	0

The antibacterial properties of the supernatant produced by the symbiotic bacteria act as inhibitors against Gram-positive bacteria and were merely bacteriostatic for Gram-negative bacteria. As gram-positive symbiotic bacteria widely know contain bacteriocins (Mezaini et al. 2009; Li et al. 2015) bacteriocins from Gram-positive bacteria are generally not effective against Gram-negative bacteria (Smaoui et al. 2010). Paper disc with supernatant applied to a Gram-positive bacterial plate indicate a stable clear zone even after a 48-hour incubation period. While against the Gram-negative bacteria, the presence of inhibitory activity appeared around the disc paper, but it was gradually turbulent before the incubation period reaches 24 hours. The antibacterial compounds produced by symbiont bacterial isolates showed different inhibitory activity against both tested bacteria *S.aureus* and *E.coli*. According to Soria-Mercado et al. (2011), the inner symbiotic bacteria generally have abundant populations and are specific because they directly interact with the bioactive compounds produced from within the algae. While the symbiotic bacteria were less populated, as it required higher defense power to overcome the pathogens and predators that are around the algae.

Antimicrobial agents may be bacteriostatic at low concentrations but are bactericidal at high concentrations (Baquero and Levin 2020). Other factors that affect the inhibition potential are the concentration or intensity of antimicrobial agents, the number of microorganisms, the temperature, the species of microorganisms, the presence of organic matter, and the degree of acidity (pH) (Manisha and Shyamapada 2011).

The area of the symptomatic supernatant inhibition zone of *S.aureus* was 6.7 mm. According to Mounyr et al. (2016), less than 10 mm inhibition zone showed weak activity and if the inhibition zone is greater than 15 mm it indicates strong activity. Testing of antibacterial activity of the symbiont bacteria supernatant obtained was still far from the results of the antibiotic activity of the chloramphenicol control. This is because of the supernatant containing secondary metabolites. However, the test results provide clear evidence of antibacterial activity. Generally, the chemical structure of metabolites from marine products differs from the terrestrial origin. Marine bacteria are significant reservoirs of bioactive molecules that have never been found in terrestrial organisms (Barzkar et al. 2019). Seawater contains an active inhibitor agent for Gram-positive bacteria (Kapoor et al. 2017).

Identification of phenotype and genotype of symbiont bacteria

The known characteristics of symbiont bacteria through phenotypic observation and biochemical tests include rod-shaped, non-acidic, non-spore-forming, non-motile, grow aerobically, negative catalase, and positive carbohydrate test In general, the selected isolate showed special characteristics possessed by lactic acid bacteria (*Lactobacillus* spp.), such as circular, smooth white, Gram-positive colonies with brief stem cells, without shaping endospores (Davoodabadi et al. 2015).

The molecular identification was done through partial genetic analysis of 16S rDNA. PCR amplification results from the 16S region of bacterial ribosome DNA Nitrogen base sequences sorted from symbiont bacterial isolates can be seen in Figure 4. The sequencing information was under the influence of genomic information enlisted within the DDBJ/Japanese DNA Information Bank with 100% strains grouping comes about. The characters of the antibacterial strains were indistinguishable from those of *Lactobacillus plantarum*. The highest 100% personality, greatest score 2660, add up to score 2660, 100% inquiry scope, E esteem 0, was recorded for the taxon of adjacent microbes. The classification of the bacterial isolate is Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*; *Lactobacillus plantarum*.

Sequens of 16S rDNA

GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAG
TGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTT
GGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCA
CCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAA
CATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGC
ATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGG
CGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAA
CGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACAT
ACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGA
GTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTC

Figure 4. The arrangement of nitrogen bases sequenced from symbiont bacteria, A: adenine, T: thiamine, G: guanine, C: cytosine

Data for base sequence encoding gene of 16S rDNA shows that symbiont bacteria have accurate scores for species levels with a similarity of 100% of the sequences present in GenBank. The species homology of the tested isolate was *L. plantarum*. *L. plantarum* strains separated from dairy items appeared solid antimicrobial action against the pointers strains of *S. aureus*, *Salmonella* spp, and *E. coli* (Hu et al. 2019). The isolation of *L. plantarum* from Tibetan yaks was able to restrain the development of *E. coli* and *S. aureus* (Wang et al. 2018). Some *Lactobacillus* strains showed antibacterial movement against Enterobacteriaceae that were safe for carbapenems (CRE). This effect may have potential applications through the utilize of the *Lactobacillus* strain as a starter culture in aged nourishments or as a nourishment additive to control or avoid CRE contamination (Chen et al. 2019).

In conclusion, *T. conoides* was commonly found in the gulf of Banten, Serang district, province of Banten. This research revealed that symbiont bacteria *L. plantarum* was endophytic and potentially useful as an antibacterial agent against common pathogens.

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