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Investigation of Seagrass-Associated Fungi as Antifouling Candidates with Anti-Bacterial Properties

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Abstract

The aim of this study was to test seagrass-associated fungi that have antibacterial activity against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Seagrass sampling, fungi isolation, antibacterial screening, and molecular identification were used in this study. The antibacterial screening found that 2 out of 10 isolates were positive for antibacterial activity. This includes a seagrass-associated fungi of *Thalassia hemprichii*, which has green colonies. This isolate has no exudates and has a green reverse. The other isolate TA.EA.1.3 was a seagrass-associated fungi of *Enhalus acoroides*, white in color, with exudates and a brown reverse. The isolate extracts weighed 256 mg and 2017.9 mg, respectively. The antibacterial activity of the TA.TH.1.1 fungal isolate extract against *V. alginolyticus* and *Vibrio parahaemolyticus* was classified as strong because of the inhibition zone produced, which was 10-20 mm. The antibacterial activity of the TA.EA.1.3 isolate, classified as moderate, produced 5-10 mm of inhibition zone. Molecular identification showed that isolate TA.TH.1.1 was *Aspergillus unguis* (100% homology) and TA.EA.1.3 was *Aspergillus versicolor* (100% homology). In conclusion, *A. unguis* and *A. versicolor* have antibacterial ability against *Vibrio alginolyticus* and *V. parahaemolyticus* as antifouling bacteria.

Keywords: Antibacteria, Antifouling, Association, Fungi, Seagrass

1. Introduction

Marine ecosystems have high biodiversity, including a large population of bacteria. One liter of seawater can contain more than 20,000 bacterial cells (Sogin et al., 2006). *Vibrio* spp. survive in warm-brackish water, even in Indonesia (Baker-Austin et al., 2018). *Vibrio* spp. bacteria can cause a lot of losses in the marine sector, by leading to, for instance, the formation of a biofilm layer on some marine organisms (Ashrafudoulla et al., 2021). In addition, marine organisms contaminated by *Vibrio* spp. can get vibriosis, which causes huge economic losses in the aquaculture (Liu et al., 2013). This species reportedly also forms biofilms on the surface of ship hulls and marine infrastructure (Waturangi et al., 2017). Antifouling technology is used in controlling biofouling, for example, biofouling paint. Antifouling often used chemicals, such as tributyltin (TBT), Cu, Zn, Ba, Cd, Cr, Ni, and Pb, which are toxic to non-target objects. As a result, International Maritime Organization (IMO) banned the use of TBT and heavy metals for antifouling in 2008 (Qian et al., 2010).

Seagrasses are plants that have adapted to a life immersed in shallow seas. These higher plants have a high tolerance to salt levels in their habitat and reproduce by seeds (Touchette, 2007). Seagrasses have different kinds of associations with various organisms, one of which is fungi. Seagrass species, such as such as *Posidonia oceanica*, *Thalassia hemprichii*, and *Halophila ovalis*, accumulate large amounts of fungi in their bodies, (Ling et al., 2015). Seagrass-associated fungi have a bioactive compound that allows their host to defend themselves from insects and pathogenic microorganisms (Gono et al., 2022). Such fungi can inhibit the formation of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* bacteria (Supaphon et al., 2013; Rabbani et al., 2021). Activities of marine-derived fungi (MDF) isolated from seagrasses had potency in antibacterial, cytotoxic, and trypanocidal and salinity may influence the bioactivities of some species of MDF (Notarte et al., 2018).

Vibrio spp. is a kind of gram-negative bacteria and is comma-shaped. This bacteria is a natural constituent in freshwater, estuary, and warm marine ecosystems (Baker-Austin et al., 2018). The key factor supporting the survival of *Vibrio* spp. in marine ecosystems is its ability to form biofilms (Yildiz & Visick, 2009). Biofilm is a collection of microbial cells attached irreversibly to a surface, both biotic and abiotic, and encased in a matrix formed from polysaccharide material (Donlan & Costerton, 2002). *Vibrio* bacteria can form biofilms on the surface of some marine organisms, such as crabs, oysters, clams, and shrimp. They can also infect marine organisms and cause diseases, both for these organisms themselves and for humans who consume these organisms (Ashrafudoulla et al., 2021). Marine organisms contaminated by *Vibrio* can get vibriosis which causes huge economic losses in the aquaculture industry (Liu et al., 2013). Humans who consume marine organisms contaminated by *Vibrio* bacteria can complain from gastrointestinal diseases with

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various symptoms, such as vomiting, headache, and nausea (Xi et al., 2012). This study aims to explore the potential of extracts of seagrass-associated fungi as an antibacterial product against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*.

2. Materials and Methods

2.1. Site Research

Samples of the seagrass species *Cymodocea serrulata, Enhalus acoroides*, and *Thalassia hemprichii* from Teluk Awur, Jepara, Central Java, Indonesia were used in this research. Fresh samples were isolated in order to maximize the number of associated fungi. The isolation process was carried out at the Integrated Laboratory, Diponegoro University. Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) were used for fungal isolation (Sibero et al., 2018). The isolated samples were then incubated and observed for seven days. Bacterial screening was carried out to determine potential isolates for antibacterial activity. The screening was carried out based on the and cultured in a nutrient agar medium at room temperature (Sibero et al., 2017).

2.2. Sample Preparation

The extraction of seagrass-associated fungi was carried out by the maceration method (Payangan, 2018). The cultured fungi were macerated using ethyl acetate (EtOAc) solvent for 72 hours, and the solvent was changed every 24 hours. The maceration results were filtered using filter paper. The remaining solvent in the filter was evaporated using a rotary evaporator with nitrogen gas.

2.3. Antibacterial activity

The agar diffusion method (or the Kirby-Bauer disc diffusion method) was used to test the antibacterial activity. The pathogens used were *V. alginolyticus* and *V. parahemolyticus*. The fungal extract was diluted with dimethyl sulfoxide (DMSO) into three different concentrations, which were 100 g/disc, 250 g/disc, and 500 g/disc. Extracts from each concentration were then diffused on paper discs (Ø 6 mm OxoidTM) as much as 10 l/disc. Chloramphenicol was used as a positive control and DMSO was used as a negative control in the test (Sibero et al., 2018).

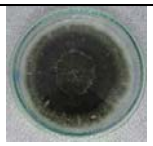
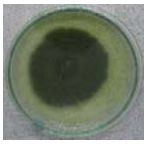
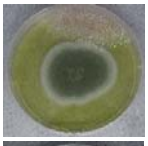
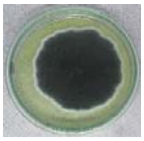
2.4. Molecular identification

DNA extraction was carried out using the Zymo® DNA Extraction Kit. Pure isolates of fungi were grown in an agar medium for seven days and used for DNA extraction. Amplification was carried out using the Internal Transcribed Spacer (ITS) as a primer for fungi barcoding when using polymerase chain reaction (PCR). The quality of PCR products was checked using electrophoresis with tris base buffer, acetic acid, and EDTA (TAE) 1X. The sequences obtained were processed using the MEGA X software and then formed a phylogenetic tree.

3. Results

The seagrasses associated with fungi, particularly *E. acoroides, T. hemprichii* and *C. serrulata*, showed several results (Table 1). There were 4 isolates of fungi isolated from C. *serrulata*, 3 isolates of fungi from *T. hemprichii,* and 3 isolates of fungi with different colors, mycelium, exudates, and reverse. Isolate TA.TH 1.1 from *T. hemprichii* had green colonies with white mycelium. This isolate had no exudates and had a green reverse. Isolate TA.EA.1.3 from *Enhalus acoroides* was white in color, white in mycelium, had exudates and a brown reverse. The screening was carried out before the isolate extract process.

Table 1: Results of Morphological Identification of Seagrass-associated Fungi

No	Seagrass	Isolate code	Results	Colony colour	Miselium	Exudates	Reverse
1	*Cymodocea serrulata*	TA.CS.1.1		black	black	No	black
2	*C. serrulata*	TA.CS.1.2		black	white	No	white
3	*C.serrulata*	TA.CS.1.3		black	white	no	brown
4	*C. serrulata*	TA.CS.1.4		white	white	No	pink
5	*T. hemprichii*	TA.TH.1.1		green	white	No	green
6	*T. hemprichii*	TA.TH.1.2		green	green	No	red
7	*T. hemprichii*	TA.TH.1.3		green -white	white	No	brown
8	*E. acoroides*	TA.EA.1.1		white	white	No	yellow
9	*E. acoroides*	TA.EA.1.2		blue	white	exist	blue
10	*E. acoroides*	TA.EA.1.3		white	white	No	brown

The screening results showed that 2 isolates of seagrass-associated fungi had the potential for antibacterial activity against *Vibrio alginolyticus* and *Vibrio parahaemolyticus* bacteria, namely isolates TA.TH.1.1 and TA.EA.1.3. The isolates were then extracted, yielding results that can be seen in Table 2.

Table 2: Seagrass Associated fungi Isolate Extract Weight

No	Isolate code	Weight (mg)
1	TA.TH.1.1	256
2	TA.EA.1.3	207.9

The TA.TH.1.1 isolate had an extract weight of 256 mg and the TA.EA.1.3 isolate had an extract weight of 207.9 mg. The results of the antibacterial activity test of the isolates of fungi-associated seagrass TA.TH.1.1 and TA.EA.1.3 against *V. alginolyticus* and *V.*

parahaemolyticus bacteria were observed over an incubation period of 24 hours and can be seen in Table 3.

Table 3: Seagrass Associated fungi Isolate Antibacterial Activity Test Results

No	Isolate Code	Concentration (µg/*disc*)	Avg. Inhibition Zone Diameter (mm)	
			V. alginolyticus	***V. parahaemolyticus***
1	TA.TH.1.1	500	15.47 ± 0.40	15.37 ± 0.19
		250	14.13 ± 0.33	12.46 ± 0.28
		100	11.10 ± 0.32	8.70 ± 0.23
2	TA.EA.1.3	500	9.02 ± 0.49	8.60 ± 0.45
		250	10.10 ± 0.48	10.19 ± 026
		100	5.8 ± 0.17	5.92 ± 0.21
3	Positive Control (Chloramphenicol)	30	15.15 ± 0.23	18.77 ± 0.24
4	Negative Control (DMSO)	-	-	-

The average diameter of the largest inhibition zone produced by isolates TA.TH.1.1 against *V. alginolyticus* was 15.47 mm at a concentration of 500 g/disc, while that produced by isolates TA.TH.1.1 against *V. parahaemolyticus* was 15.37 mm at a concentration of 500 g/disc. The average diameter of the largest inhibition zone produced by isolate TA.EA.1.3 against *V. alginolyticus* was 10.10 mm at a concentration of 250 g/disc, and by isolate TA.EA.1.3 against *V. parahaemolyticus* was 10.19 mm at a concentration of 250 g/disc. Potential isolates that had been amplified using PCR were characterized by the formation of a single band from each fungal DNA. Gene amplification results were used for further identification. The results of visualization of the DNA of fungi isolates TA.TH.1.1 and TA.EA.1.3 can be seen in Figure 1.

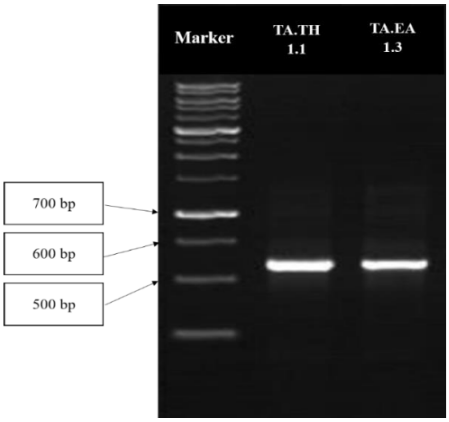


Figure 1: Result of Isolates TA.TH.1.1 dan TA.EA.1.3 DNA Visualization

The DNA visualization was followed by a homology BLAST search. According to the results obtained, isolate TA.TH.1.1 had a close kinship level (100%) with *Aspergillus unguis* (Acc Number MT582759.1), while isolate TA.EA.1.3 had a very close relationship (100%) with *Aspergillus versicolor* (Acc Number MK140698.1).

Table 4: Homology BLAST Search Results

No	Isolate Code	Identification Result	Query Cover	Percent Identify	E Value	Acc Number
1	TA.TH.1.1	*Aspergillus unguis*	100%	100%	0.0	MT582759.1
2	TA.EA.1.3	*Aspergillus versicolor*	100%	100%	0.0	MK140698.1

The results of processing base pair data for making a phylogenetic tree can be seen in Figure 2.

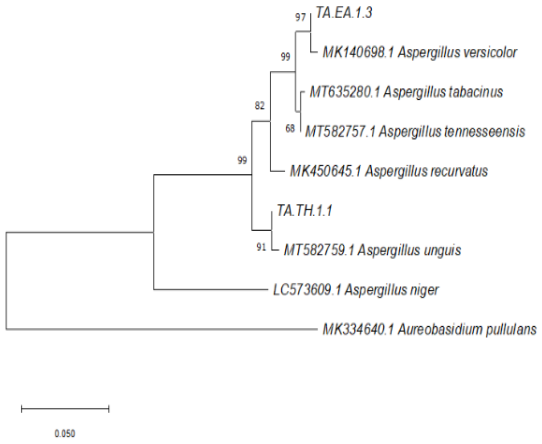


Figure 2: Phylogenic tree of TA.TH.1.1 and TA.EA.1.3 fungi isolate

The results of data processing showed that there was a clustering of seagrass-associated fungi isolates with other organisms in the closest to farthest kinship levels. The level of kinship in the isolate TA.TH.1.1 showed a very close relationship with *Aspergillus* unguis while the isolate TA.EA.1.3 showed a very close relationship with *Aspergillus versicolor*. The out group used was the fungus *Aerobasidium pullulans* (MK334640.1) because it is a species that has no close relationship with the fungi isolates TA.TH.1.1 and TA.EA.1.3.

4. Discussion

The obtained results showed 7 isolates on PDA media and 3 isolates on MEA media. Isolation code naming and morphological characteristics PDA and MEA media were used in the growth of associated fungi. PDA increases fungi sporulation and pigment production (Fitriana et al., 2018). The MEA medium was used to grow fungi mycelia and to perfect its morphological characters (Abdullah &

Saadullah, 2018). The TA.TH.1.1 fungal isolate extract was heavier than the TA.EA.1.3 yeast isolate extract, due to the different media used, which led to differences in spore growth. This statement is supported by Abdullah and Saadullah (2018), who show that the PDA medium is generally used to accelerate fungi sporulation.

The results showed that the extract with a high concentration showed better antibacterial activity against the test bacteria. The antibacterial activity was high against microbes if the minimum inhibitory concentration is low but the inhibitory power is high (Balouiri et al., 2016). The antibacterial activity of the extract of the fungal isolate TA.TH.1.1 against the bacteria *V. alginolyticus* and *V. parahaemolyticus* was strong, while the antibacterial activity of the extract of the fungus isolate TA.EA.1.3 against the bacteria *V. alginolyticus* and *V. parahaemolyticus* was moderate. According to Davis and Stout (1971), an inhibition zone with a diameter of >20 mm can be categorized as having very strong activity.

The results showed that the isolate TA.TH.1.1 had a very close relationship with the fungus *Aspergillus unguis* with a query cover of 100% and homology (percent identify) of 100%. Isolate TA.EA.1.3 had a very close relationship with the fungus *Aspergillus versicolor* with a query cover of 100% and homology (percent identify) of 100%. The results of the analysis obtained are categorized as similar because they have a similarity percentage above 97%. As Hagström et al. (2000) have stated, isolates that had a similarity percentage of more than 97% could be said to represent the same species.

5. Conclusions

Fungi-associated seagrasses have antibacterial potential against *V. alginolyticus* and *V. parahaemolyticus* that cause fouling. *Aspergillus unguis* and *Aspergillus versicolor* were identified by molecular identification based on ITS region sequences and were known to form inhibition zones against *V. alginolyticus* and *V. parahaemolyticus.*

Contribution of authors

All authors are equal in contribution to the paper.

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