Potential of associative bacteria isolates from seagrass ecosystem

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Abstract. The seagrass ecosystem is rich in organic materials such as nitrates, carbon, and phosphates. Potential nutrient content available in water and sediments influences the presence of an associative bacterial community. This study aimed to explore associative bacteria in seagrass ecosystems which have potentially producing antibacterial activity and cellulase enzymes. Associative bacterial isolation was carried out on sediment samples and seagrass leaves Thalassia hemprichii which was found as the dominant ecosystem compiler. The antibacterial test was carried out using agar diffusion method against Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, and Vibrio parahaemolyticus. Meanwhile, assay on cellulase activity of the actinomycetes bacteria was using agar diffusion method on CMC media. Isolation of endophytic bacteria of seagrass leaves produced 12 isolates with 2 isolates which could inhibit all targeting bacteria and 3 isolates which could inhibit several targeting bacteria. ANOVA test showed that the sampling location significantly affected the antibacterial activity. Meanwhile, 5 isolates of actinomycetes bacteria were found from the sediments, with 4 isolates having cellulase activity. ANOVA test showed that cellulase activity of the actinobacteria was associated with the density of Cymodocea rotundata and T. hemprichii seagrass species with moderate to high category.

1. Introduction

Seagrass is flowering plants (angiosperms), has rhizome roots, true leaves and roots. Seagrass plants can adapt to waters with high salinity [1]. Seagrass ecosystems have an important role to maintain the balance of the waters because they can be found harboring many species of marine biota and endophytic microorganisms. Endophytic microorganisms can produce bioactive compounds that have the potential to be developed in the pharmaceutical, industrial, and agricultural fields [2]. The resulting bioactive compounds are called secondary metabolites. The production of secondary metabolites functions as a defense in the environment [3].

Some chemical components derived from plants are used as medicines such as catechins, genistein, flavanoid, and stylebenoids [4]. Endophytic bacteria isolated from plants can produce secondary metabolites that are the same as the original plants in even higher amounts [5]. Whereas, actinomycetes produce more than 50% antibiotic compounds [6]. Seagrass ecosystems are rich in organic materials such as nitrates, carbon, and phosphates. Potential nutrient content available in waters and sediments influences the presence of associative bacterial communities. This study aimed to explore associative bacteria in seagrass ecosystems which have potentially producing antibacterial activity and cellulase
enzymes. Associative bacterial isolation was carried out on sediment samples and seagrass leaves *Thalassia hemprichii* which was found as the dominant ecosystem compiler.

2. Method

2.1. Seagrass density and sample taking the process
Seagrass data collected using the seagrass watch method [7] in Pari Island waters and the density of seagrass was calculate based on the ratio between total seagrass individual and area of the transect (m²) [8]. Seagrass tissue that collected as a sample was *Thalassia hemprichii*, as the dominant seagrass species. It collected in 0.5-1 m depth, put inside a sample plastic, gave label and preserved it in cold condition [9]. Seagrass sediment ecosystem took by sediment core, put it inside sample plastic and preserved in cold condition [10].

2.2. Isolation and purification process
Isolation from seagrass samples started by cleaned the seagrass leaves under flowed water to remove attached particles on the leaves. Leaves then cut into 5 mm, sterilized using alcohol 70%, washed using sterile water, tore it and put the inside part of the leaf on Zobell 2216E agar [11]. Incubated them for 3-4 days in 30°C and did observation every day [12]. Bacteria purification using Zobe 2216E agar was done after the appearance of the bacteria colony.

Actinomycetes bacteria from sediment were isolated and purified using *Yeast Extract Malt Extract Agar* (ISP 2). Sediment was dried on 27°C for a night [13] then mixed with sterile seawater and diluted it until 10⁻³. Isolation using 10⁻² and 10⁻³ dilution into sterile ISP 2 which added by nystatin and nalidixic acid or levofloxacin. The incubation process took 7-24 days in 28°C. After that, isolated was purified using the same media as isolation.

2.3. Antibacterial assay
Target bacteria used in the antibacterial assay were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Vibrio parahaemolyticus*. The assay with endophytic bacteria using a double layer method [15] and for actinomycetes using block agar method [16].

2.4. Cellulase assay
Cellulase assay conducted by incubated actinomycetes onto CMC 1% agar. After actinomycetes grew well on the media, dropped some iodine onto media and wait until clear zone formed. The cellulolytic index can be calculated using a ratio between a clear zone and diameter of the actinomycetes colony [17].

2.5. ANOVA and CA
Analysis of variance (ANOVA) was used to know about the connection between some parameters in population or test plot [18]. Two way ANOVA was used to know the connection between antibacterial activity of endophytic bacteria and seagrass ecosystem conditions. Meanwhile, one way ANOVA used to know the connection between cellulase activity of ecosystem and seagrass density. Another analysis that used to know about the connection between antibacterial activity of endophytic bacteria and ecosystem was correspondence analysis (CA). This analysis is a factorial analysis to grouped some units into homogenous teams.

3. Results and discussion

3.1. Seagrass density
The observations showed that the total seagrass density was different for each station. Station 1 and Station 2 had total density values respectively 144 ind/m² and 196 ind/m². The seagrass species were *Cymodocea rotundata*, *Thalassia hemprichii* and *Enhalus acoroides*. The species density value at Station 1 was *C. rotundata* 22 ind/m², *T. hemprichii* 88 ind/m² and *E. acoroides* 34 ind/m². The species
density value at Station 2 was *C. rotundata* 110 ind/m², *T. hemprichii* 40 ind/m² and *E. acoroides* 46 ind/m².

![Figure 1. Seagrass density in Pari Island (Cr= *C. rotundata*, Th= *T. hemprichii* and Ea= *E. acoroides*).](image)

The base substrate at Station 1 was classified as muddy sand, while at Station 2 was rocky sand. Different types of substrates affect the seagrass communities that exist in an area both in structure and shoots [1]. Seagrass plants that submerged in water can serve as a habitat for microbial communities, that provide nutrition to support the growth of microbial communities [19]. Seagrass cover can also act as a limiting factor for microbial growth in the water column by absorption of carbon and nitrogen [20].

### 3.2. Endophytic bacteria

Isolation of endophytic bacteria from *T. hemprichii* seagrass produced 12 isolates and was tested against 5 types of targeted bacteria. The antibacterial activity was seen from the inhibition zone around the isolates. The inhibition zone of endophytic bacteria is shown in Table 1.

| Isolates code | The diameter of inhibition zone (mm) |
|---------------|-------------------------------------|
|               | *S. aureus* | *P. aeruginosa* | *B. subtilis* | *E. coli* | *V. parahaemolyticus* |
| ThA1.1        | -           | -               | -             | -         | -                     |
| ThA1.2        | -           | -               | -             | -         | 2.14                  |
| ThA1.3        | 5.14        | 2.96            | 4.16          | 4.41      | 2.27                  |
| ThB1.1        | -           | -               | -             | -         | 2.13                  |
| ThB1.2        | 2.23        | 6.45            | -             | -         | -                     |
| THB3.1        | -           | -               | -             | -         | -                     |
| THC1.1        | 4.37        | -               | -             | 4.34      | 2.16                  |
| THC1.3        | 7.97        | 5.5             | 4.01          | 3.28      | 1.75                  |
| THC2.1        | 4.27        | 2.23            | 3.22          | -         | 1.83                  |
| THC2.3        | -           | -               | 4.06          | -         | 1.74                  |
| THD3.1        | -           | -               | -             | 1.85      | 1.66                  |
| ThD3.2        | -           | -               | -             | -         | 2.05                  |

The range of inhibition zone produced by all isolates was 1.66-7.97 mm. Table 1 showed that 9 isolates had activity on inhibiting the growth of targeted bacteria, and 3 isolates did not show any activity against all targeting bacteria. The most potential isolates were ThA1.3 and ThC1.3 with the ability to
inhibit the growth of all targeting bacteria. Figure 2 showed the antibacterial activity by ThA1.3 isolate against all targeting bacteria.

Figure 2. Inhibition ability of ThA1.3 isolate against (from left to right) S. aureus, P. aeruginosa, B. subtilis, E. coli and V. parahaemolyticus [9].

The antibacterial activity exhibited by T. hemprichii endophytic bacterial indicates that there are secondary metabolite compounds produced by these bacteria. Some active compounds found in seagrass include tannins, flavonoids, saponins, terpenes, alkaloids and glycosides which are antimicrobial [21]. The mechanism of antibacterial compounds is generally performed by damaging cell walls, changing membrane permeability, inhibiting enzyme work and interfering with protein synthesis [22].

The antibacterial activity than were associated with the targeting bacteria, as well as the seagrass density. The analysis showed that the antibacterial activities of endophytic bacteria were centered on axis 1 and axis 2 with a total range of 67.35% (Figure 3). Whereas 4 isolates of endophytic bacteria (ThA1.2; ThC1.3; ThD3.1 and ThD3.2) had a strong correlation with 2 targeting pathogenic bacteria (VP and PA, respectively), and 2 isolates of endophytic bacteria (ThC1.1 and ThD2.3) had a strong correlation with another two targeting pathogenic bacteria (EC and BS). This result implies that most of the endophytic bacteria have a strong correlation with biofilm-forming bacteria, such as V. parahaemolyticus, P. aeruginosa and B. subtilis [23] found that primary biofilm-forming bacteria that live on the surface of Halodule pinifolia seagrass leaf dominated by Staphylococcus, Pseudomonas, E coli and Bacillus bacteria. This also supported by [24] that worked on antibacterial activity from seagrass against biofilm bacteria. The ability of seagrass endophytic bacteria to inhibit the biofilm-forming bacteria suggest that the endophytic bacteria may have a role in host-plants biocontrol against epiphytic mechanism in the environment. Overall, correspondence analysis showed that the antibacterial activity of endophytic bacteria only correlates as much as 67.35% with the targeting bacteria. Hence, it implies that there is another factor could influence the activity of antibacterial compounds from the endophytic bacteria.

Figure 3. CA between endophytic bacteria and targeting bacteria (SA= S. aureus, PA= P. aeruginosa, BS= B. subtilis, EC= E. coli, and VP= V. parahaemolyticus).
Differences in antibacterial activity also can be related to the seagrass sampling location. Seagrass samples were taken on quadratic transects to 0 m and 50 m. Based on the ANOVA test results, the relationship between the sampling location and the antibacterial activity of the two was significantly different (p<0.05) (Table 2). Thus, it suggests that antibacterial activity will significantly differ in each location, although it came from an adjacent location on the same island. It is suspected that the abundance of organic matter in the sampling location will affect the biotic and abiotic microenvironment in the ecosystem.

Oceanfront areas contain more nutrients than in the open ocean, thus it can support the growth of various microorganisms. Subsequently, this may result in a dense population of microorganisms which furthermore can stimulate the growth of chemoautotrophic bacteria, fish, and shellfish [25]. However, the water quality in both transects should not be much different because the distance between the transects was close, so the seagrass density factor on the second transect data collection is suspected to have more influence on antibacterial activity. As mentioned by [26], that ephyphic heterotroph-bacteria abundance will be higher corresponds to the density of seagrass shoots.

Table 2. ANOVA analysis on the relationship between antibacterial activity with the location of sampling and seagrass density.

| Variables | Factors                      | Df  | F     | P-value | Fcrit |
|-----------|-------------------------------|-----|-------|---------|-------|
| Sampling location | Location | 1   | 8.685 | 0.005   | 4.034 |
|          | Targetting bacteria          | 4   | 0.335 | 0.853   | 2.557 |
|          | Location * Targetting Bacteria | 4   | 1.009 | 0.412   | 2.557 |
| Seagrass Density | Density | 1   | 2.299 | 0.136   | 4.034 |
|          | Targetting bacteria          | 4   | 0.287 | 0.885   | 2.557 |
|          | Density * Targetting bacteria | 4   | 0.324 | 0.860   | 2.557 |

However, the ANOVA test showed that seagrass density has a significant relation with the antibacterial activity of the endophytic bacteria, with p> 0.05 (see Table 2). This possibly happened for there were only slight differences in the seagrass density in both observation stations (Station 1 and Station 2). Another reason for this insignificant value was there is a possibility that the observation was only conducted from 0 m and 50 m of transects (at both edges of transects), thus were not represented the seagrass density, accurately.

3.3. Actinomycetes

Isolation of actinomycetes from seagrass sediment produced 5 isolates and was tested on 4 types of targeted bacteria. The result of the antibacterial assay showed that only one isolate, B22b, which has an inhibition zone with a range of 8-13 mm [10], as showed in Figure 4. The inhibition zone that appeared in the antibacterial assay was a mechanism of actinomycetes to inhibit the growth of other bacteria by the secreted antibacterial compound. The larger inhibition zone formed in the assay indicates that the antibacterial compound secreted by actinomycetes was higher [28]. The B22b isolate can inhibit Gram-negative as well as Gram-positive bacteria, thus it was estimated the isolates has a broad spectrum activity based on its activity [28].

Figure 4. Result of the antibacterial assay with (a) B. subtilis, (b) E. coli, (c) P. aeruginosa and (d) S. aureus as targeting bacteria against (1) A11a, (2) B22b, (3) A10b and (4) A30a as actinomycetes isolates.
The B22b isolates showed different inhibition activity against *S. aureus* and *B. subtilis* through both bacteria are Gram-positive. This isolate was cultured from sediment that has a higher density of *C. rotundata* than *T. hemprichii* (Figure 1, Station 2). Therefore, it suggests that actinomycetes isolate that cultured from sediment plants root could have different activity [29].

Based on its inhibition zone, the B22b isolate can be categorized into low inhibition of antibacterial activity [16]. Inhibition zone affected by some factors, aside then, the antibacterial compound. Other factors that affected the inhibition zone are the sensitivity of targetting organisms, incubation conditions, and culture media [30].

Furthermore, actinomycetes were tested in cellulase assay. This assay showed that isolates with cellulase activity will have a clear zone after iodine dropped onto CMC media. Figure 5 showed the result of actinomycetes cellulase assay. The larger appearance of clear zone diameter indicated the higher activity of cellulase produced by the isolate [31]. Figure 5 showed that only 4 of 5 isolates produced a clear cellulase zone. As mentioned by a previous study [32], some actinomycetes could have no activity if cellulase enzyme.

Figure 5. Cellulase assay of actinomycetes isolates, (a) A10b, (b) A11a, (c) B22b, (d) A30a and (e) B22c.

Table 3. Cellulolytic index from actinomycetes.

|       | A11a | A10b | A30a | B22b | B22c |
|-------|------|------|------|------|------|
| Index | 1.5  | -    | 5.2  | 5.4  | 1.5  |

The clear zone of cellulase activity then converted into the cellulolytic index, which showed in Table 3. Variation of the cellulolytic index could be affected by its ecological role in the natural habitat. Some isolates (A11a, A10b, and A30a) that were cultured from the sediment with a lower density of *C. rotundata*, meanwhile other isolates originated from the denser *T. hempricii*. All isolates showed a high variety of cellulolytic index. The variation of the cellulolytic index also shown by terrestrial sediment actinomycetes from different vegetation of plants [33].

In comparison with another research [34, 17], implies the cellulolytic index could have differed values regarding the distinctive of the culture nutrition. Different nutrition ingredients of the culture media could affect the cellulolytic activity of actinomycetes. Nutrition itself is one of the external factors that affected the cellulolytic index value [17].

Table 4. ANOVA test result of seagrass species density and cellulase activity.

| Seagrass species density | F    | Sig. F |
|-------------------------|------|--------|
| C. rotundata            | 0.014| 0.912  |
| T. hemprichii           | 0.538| 0.504  |
| E. acoroides            | 0.623| 0.474  |
The relation between cellulase activity and seagrass density was analyzed using the ANOVA test, which showed in Table 4. The result showed that the cellulase activity was influenced by the seagrass density of *T. hemprichii* and *E. acoroides*. It was proposed that seagrass species, which have bigger leaves than *C. rotundata*, will produce more litter based on the statement that 80% of *T. hemprichii* leaves will be littered [35]. Increasing in plants litter correspondingly will increase the cellulose resources in the ecosystem. Consequently, actinomycetes in the sediment could take part as cellulose decomposer agents in the ecosystem.

4. Conclusion
In conclusion, seagrass endophytic bacteria showed antibacterial activity, especially against targetting bacteria with biofilm-forming ability. Sampling location (i.e distance to the land) affected the antibacterial activity of endophytic bacteria. Meanwhile, actinomycetes isolated from the sediment of the seagrass ecosystem showed more potential on the cellulolytic activity instead of the antibacterial activity. Seagrass species and shoots density have a strong influence on the cellulolytic activity, although it has shown a variation on the cellulolytic index of the actinomycetes isolates.

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