

Antifouling Activity of Bacterial Symbionts of Seagrasses against Marine Biofilm-Forming Bacteria

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ABSTRACT

Marine biofouling has been regarded as a serious problem in the marine environment. The application of TBT and other heavy metal-based antifoulants has created another environmental problem. The present study explored the possible role of bacterial symbionts of seagrasses *Thalassia hemprichii*, and *Enhalus acoroides*, which were successfully screened for antifouling activity against marine biofilm-forming bacteria isolated from the surrounding colonies of seagrasses. Bacterial symbionts were isolated and tested against biofilm-forming bacteria resulted in 4 bacterial symbionts capable of inhibiting the growth biofilm-forming isolates. Molecular identification based on 16S rRNA gene sequences revealed that the active bacterial symbionts belonged to the members of the genera *Bacillus* and *Virgibacillus*. Further tests of the crude extracts of the active bacterial symbionts supported the potential of these symbionts as the alternative source of environmentally friendly marine antifoulants.

Keywords: Biofouling, Antifoulant, Bacterial Symbionts, Seagrasses

1. Introduction

A regularly observed phenomenon, marine biofouling, is not only a natural process as a result of organism growth on underwater surfaces [1], but is also undesirable due to massive economic losses to marine industries. Primary biofilm, another phenomenon and is part of marine microfouling creates further threat by facilitating the attachment and metamorphosis of fouling organisms [2]. Bacteria as part of the marine microbial population, have been considered as the primary colonizers within biofilm and play a role as dominant components [3]. It is then reasonable to manipulate the role of these bacterial film by disrupting their growth by applying marine antifoulants.

Along with long-term application of tributyl-tin (TBT)-based antifouling paints, there has been an increased concerns due to the increased concentrations resulted in environmental hazards to marine marine ecosystems. It was then the reason for EU countries in 1989, followed by International Maritime Organization (IMO) and the

Maritime Environment Protection Committee (MPEC) to restrict the use of TBT [4]. Since then, the development of alternative environmentally friendly antifoulants has been carried out to find alternative replacements.

Sea grasses are one of the most productive coastal ecosystems and a rich source of secondary metabolites with ecologically important roles such as preventing surface fouling [5]. As an effort to explore the potential of marine antifoulants produced by seagrasses [6], it was reported the potential of seagrasses *Cymodocea serrulata* and *Syringodium isoetifolium* in inhibiting the growth of marine biofilm-forming bacteria. This finding has confirmed the possible manipulation of biofilm-forming bacteria in reducing the effects of marine biofouling.

The fact that the problem of supply has hampered the development of most secondary metabolites from marine organisms and plants, thus, it is important to highlight the possible role of marine bacteria associated with seagrasses in providing an alternative to the commercial metal-based antifouling coatings. Bacteria-seagrass association that occurs on the seagrass surface then could be

of great interest to search for potential use as commercial antifoulants.

In this work, we report the potential of marine bacteria associated with seagrasses *Thalassia hemprichii*, and *Enhalus acoroides* for controlling the growth marine biofilm-forming bacteria. Antibacterial assays of the bacterial symbionts and their crude extracts confirmed the potential role of these symbionts as the source of environmentally friendly marine antifoulants.

2. Materials and Methods

2.1. Sampling and Isolation of Bacterial Symbionts of Seagrasses

Colonies of seagrasses *Thalassia hemprichii*, and *Enhalus acoroides* were collected from seagrass beds in the Teluk Awur waters, Jepara, Central Java, Indonesia (**Figure 1**) by hands. Upon collection seagrass colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA). The colonies were then rinsed with sterile seawater and scraped off with a sterile knife for the isolation of epiphytes. As for isolation of endophytes, the surfaces of seagrass leaves were sterilized with 70% alcohol, and the leaves were cut to open the inner parts. The inner surfaces were scraped off with a sterile cutter. The resultant tissues were serially diluted, spread on 1/2 strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. Purification and isolation of bacterial colonies were performed by using streak plates on the basis of morphological features [7].

2.2. Isolation of Marine Biofilm-Forming Bacteria

Isolation was carried out according to a modified method [8]. Four pre-sterilized wooden slides had been deployed in 4 different directions around each seagrass colonies for a week. The biofilm developed in these wooden slides were then aseptically rinsed with sterile seawater, scrapped off with a sterile knife and diluted. One hundred μl of each dilution was spreaded onto 1/2 strength ZoBell 2216E and incubated at room temperature for 48 hours. Colonies with distinguished feature were selected and purified.

2.3. Extraction of Active Bacterial Symbionts

Each of active bacterial symbiont was grown in a 500 ml flask containing ZoBell2216E broth medium and incubated for 4 d in a shaker. The cultures were then centrifuged at a speed of 12,000 rpm for 5 minutes and the supernatants were collected. Supernatants were then extracted based on liquid-liquid extraction on separatory funnel (4 supernatant: 1 n-hexane). The extracts were concentrated by using a rotary evaporator and kept in freezer until antifouling activity was performed.

2.4. Antifouling Activity Test

Antifouling test of bacterial symbionts of seagrasses against marine biofilm-forming bacteria was performed by using an overlay method. Culture of each marine biofilm-forming bacterium in the logarithmic phase (ca. 10^9 cells- ml^{-1}) was

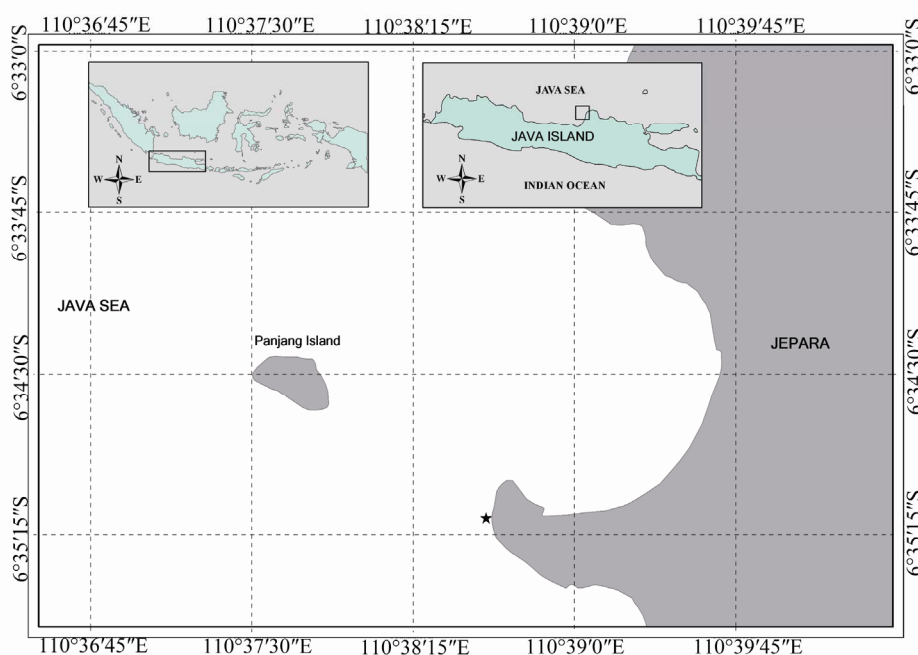


Figure 1. Sampling site at Teluk Awur, Jepara, Central Java, Indonesia.

mixed with ZoBell 2216E soft agar medium (1% v/v), which were then poured on to the respective agar surface previously inoculated with bacterial symbionts and incubated for 4 d. The plates were then incubated at room temperature for 48 hours. As for bacterial extracts, an agar diffusion method [9] was performed. Twenty microliters of each extract was poured onto a paper disk (8 mm, Advantec, Toyo Roshi, Tokyo, Japan) previously put on agar surfaces containing each biofilm-forming bacterium. Antibacterial activity was defined by the formation of inhibition zones around the bacterial colonies.

2.5. PCR Amplification and Sequencing of 16S rRNA Gene Fragments

PCR amplification of partial 16S rRNA gene of active strains and marine biofilm-forming bacteria, purification of PCR products and subsequent sequencing analysis were performed [9]. The determined DNA sequences of strains were then compared for homology to the BLAST database. A phylogenetic tree was constructed using maximum-likelihood analysis and Phylogenetic analysis was performed with the PAUP software package [10].

2.6. Nucleotide Sequence Accession Number

The 16S rRNA gene sequences of the active bacterial symbionts of seagrasses have been deposited into the DNA Database Bank of Japan with the following accession numbers: AB665240-43.

3. Results and Discussion

This work represents an effort towards finding alternative solution to the problem of marine biofouling, a serious problem faced by worldwide maritimes industries [11] and leads to huge economic losses [12].

Four bacterial symbionts of seagrasses were found to inhibit the growth of marine biofilm-forming bacteria ranging from 1 - 6 bacteria (**Table 1**). Out of 4 active isolates, 3 isolates were obtained from seagrass *E. coroides* and 1 isolate was from seagrass *T. hemprichii*. Interestingly, the crude extracts of these active bacterial symbionts showed more pronounced growth inhibition with a range of 6 - 14 bacteria (**Table 1**).

The potential of seagrass species of *Cymodocea serrulata* and *Syringodium isoetifolium* collected from the coastal area of Tuticorin has confirmed the biological activity of these seagrasses against biofilm-forming bacteria [6]. It is very reasonable that biofilm-forming bacteria have been the primary target for controlling biofouling due to their important cues for larval settlement and development of biofouling organisms [13]. However, considering the issue of bottleneck of most marine natural products and the need of sustainable use of coastal ecosystems as well as the concerns regarding the colle-

ction marine resources for discovery and development of marine natural products [14], has turned marine microorganisms such as marine microbial symbionts as a potent source of marine antifoulings. Unfortunately, less data are available of the antifouling compounds produced by marine bacteria [15-17].

The present work show the biological activity against marine biofilm-forming bacteria from the surrounding colonies of these seagrass species that were exposed into the same ecological conditions. Both endophyte and epiphytes were found to inhibit the growth of marine biofilm-forming bacteria. Interestingly, all extracts from the active isolates showed broad spectrum of antifouling activity against marine biofilm-forming bacteria. This finding is very important in regards to the fact that bacteria are the primary colonizers and play significant roles in the mediatory effects on the settlement and development of biofouling [13,18]. The higher number of biofilm-forming bacteria inhibited by the extracts of active symbionts may indicate the chemical diversity of secondary metabolites produced by the bacterial symbionts. In addition, the fact that non-polar bacterial extracts were found to be active, is a highly desirable in term of possible application of in the marine environment so that it won't be washed out easily. Furthermore, the work also confirmed the bacterial symbionts of seagrass species *T. hemprichii* and *E. acoroides* provide sustainable use of seagrass ecosystems in term of developing a large-scale cultures of bacterial symbionts for possible commercial development.

The active bacterial isolates were closely related to the members of genus *Bacillus* and *Virgibacillus* with highest homology of 98% - 99% (**Table 2**). Molecular phylogenetic (**Figure 2**) shows that all active isolates belonged to the family Bacillaceae. It is revealed that isolates EA2.1 and EA.6 belong to *Bacillus aquamaris* and *Bacillus* sp., respectively. On the other hand, isolates ESJ.5 and TT.9 belong to *Virgibacillus olivae* and *Virgibacillus marismortui*.

A report on the biological activity of bacterial symbionts of sponge *Pseudoceratina purpurea* shows that they actively inhibited the growth of fouling bacteria isolated from natural marine biofilm. The 3 active isolates belonged to genus *Bacillus* and 1 isolate belonged to *Virgibacillus* and were also found to show a broad spectrum biological activity against marine biofilm-forming bacteria [19].

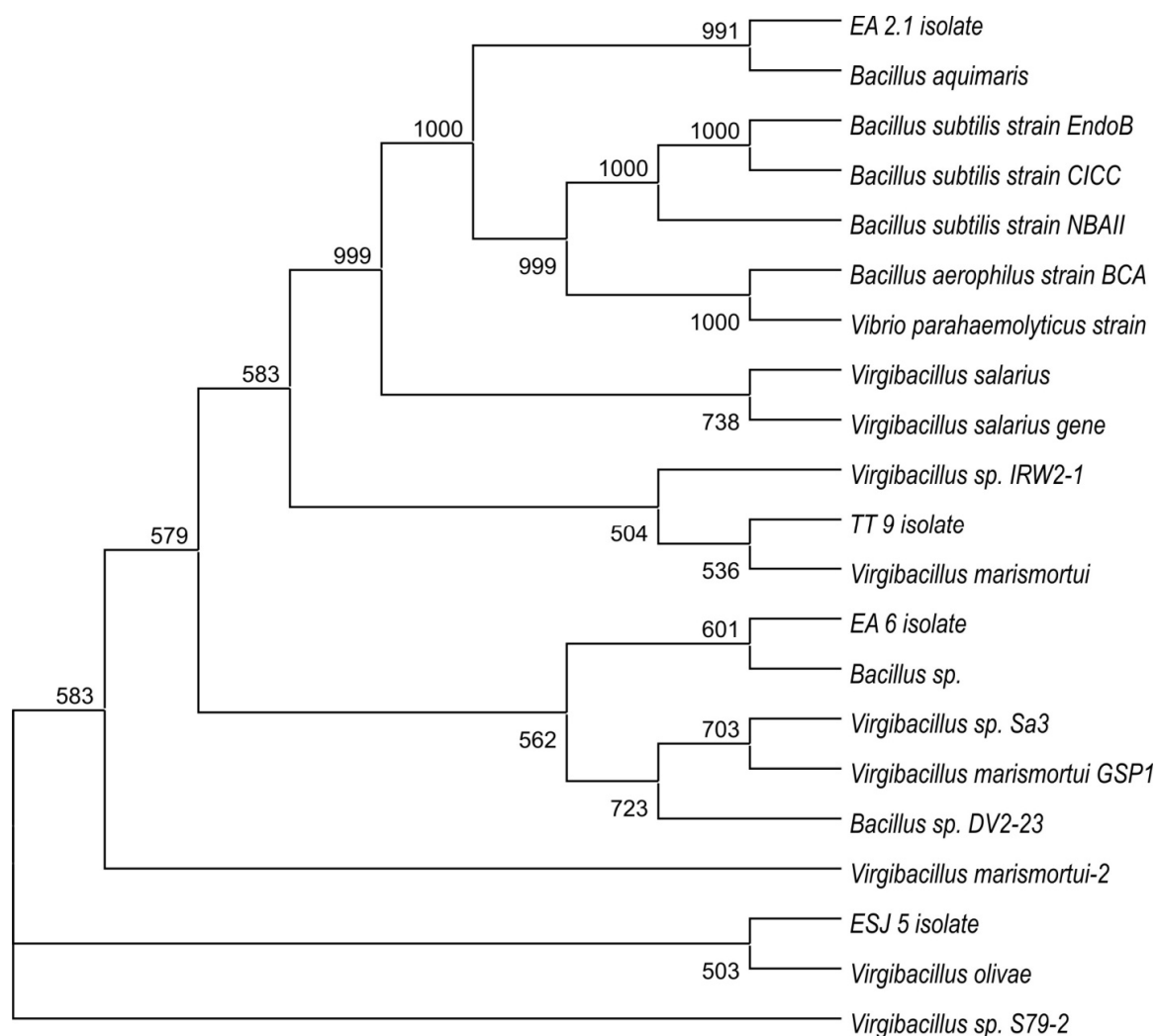
In another study, various marine bacteria associated with a range of marine surfaces were screened and showed potential application as natural antifoulants and found that the isolates were related to *Bacillus mojavenensis* and *Bacillus firmus* [18]. A screening on bacterial symbionts of softcoral *Sinularia* sp. and it was found

Table 1. Growth inhibition of biofilm-forming bacteria by bacterial symbionts and their crude extracts.

No	Isolate	Type of symbiont	No of biofilm bacteria inhibited by bacterial symbiont	No of biofilm bacteria inhibited by extract of symbiont
1	EA2.1	Ephyphite	3	8
2	EA.6	Ephyphite	1	14
3	ESJ.5	Endophite	6	6
4	TT.9	Ephyphite	2	9

Table 2. Molecular identification of active bacterial symbionts of seagrasses.

No	Isolate	Source	Closest relative	Similarity (%)	Accession No.
1	EA2.1	<i>E. acoroides</i>	<i>Bacillus aquamaris</i>	99	EU443752
2	EA.6	<i>E. acoroides</i>	<i>Bacillus</i> sp.	98	HQ704707
3	ESJ.5	<i>E. acoroides</i>	<i>Virgibacillus olivae</i>	99	HM179216
4	TT.9	<i>T. hemprichii</i>	<i>Virgibacillus marismortui</i>	99	GU172145

**Figure 2. Phylogenetic affiliation of bacterial symbionts of seagrasses.**

that the member of genus *Virgibacillus* inhibited the growth of MDR strain *Staphylococcus aureus* [19].

In conclusion, the present study represents the potential of bacterial symbiont of seagrass species *T. hemprichii* and *E. acoroides* as the alternative source of environmentally friendly marine antifoulant. The so far uncharacterized secondary metabolites from these active bacterial symbionts deserve further work on the bioassay-guided isolation and purification of the active antifouling compounds.

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