ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

Original Paper

SCREENING OF BACTERIAL SYMBIONTS OF SEAGRASS *Enhalus* sp. AGAINST BIOFILM-FORMING BACTERIA

Bintang Marhaeni[1], Ocky Karna Radjasa [2], Dietriech G. Bengen[1] dan Richardus.F.Kaswadji[1]*

[1] Graduate School of Marine Science, Bogor Agricultural University, Bogor, Indonesia
[2] Department of Marine Science, Diponegoro University, Semarang 50275, Central Java, Indonesia

Received : January, 3 th 2010 Accepted : January, 29th 2010

ABSTRACT

Seagrasses have been known to produce secondary metabolites that have important ecological roles, including preventing from pathogen infections and fouling organisms. A research aimed at screening the potential of bacterial symbionts of seagrass Enhalus sp. was performed. Bacterial symbionts including endophytes and epiphytes were isolated from the seagrass, and marine biofilm-forming bacteria were isolated from the fiber and wooden panels from the surrounding colonies. A total of 17 epiphyte and 6 endophyte isolates were obtained, however more biological activity was found among endophytes (100%) compared to epiphytes (47%) against biofilm-forming bacteria. In addition, bacterial endophytes inhibited more biofilm-forming bacteria than epiphytes. Interestingly more isolates were obtained from rough surfaces both from fiber and wooden panels than smoothe surfaces. Bacterial symbionts of seagrass Enhalus sp., in particular its endophytes show potential source as natural marine antifoulants.

Key words: bacterial symbionts, epiphytes, endophytes, *Enhalus* sp.

Correspondence: Phone: +62-251-622961-; Fax: +231-251-622986 ; E-mail: bmarhaeni@yahoo.com

INTRODUCTION

Biofouling is defined as the attachment and metabolism of microorganisms (microbial fouling) and macroorganims (macrofouling) to solid surfaces and is usually considered detrimental. Biofouling marine bacteria are the first type of organism to populate a surface placed in the marine environment and normally constitute the predominant organisms in most biofilm, thereby comprising the first stage of a progression that leads to a complex macrofouling community (Smitt, 1988). Surface films of marine fouling bacteria appear to play a role in the settlement, attachement and metamorphosis of some marine invertebrate larvae (Maki and Mitchell, 1988).

Marine biofouling, despite a natural process as a result of organism growth on underwater surfaces (Pereira *et al.,* 2002), causes huge economic losses to marine industries. In seawater, the microbial population on surface produce primary biofilm, which is generally thought to be a prerequsite for the attachment and metamorphosis of fouling organisms (Callow and Callow, 2002).

 ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

One of the most productive communities on earth, the sea grass community is found in estuaries, lagoons, and shallow open shelves. The sea grasses are important component of the coastal ecosystems that provide nursery grounds for commercially fish and invertebrate species, stabilize sediments and fixes significant of inorganic carbon (Jensen et al, 1998). Sea grasses are a rich source of secondary metabolites (McMillan *et al.,* 1980), and have ecologically important roles similar to allelopathic agents (Zapatta and McMillan, 1979). Given the potential threats imposed by marine pathogens and epiphytes, marine plants produce secondary metabolites that prevent them from bacterial infection and surface fouling.

Many secondary metabolites produced by marine plants and invertebrates are thought to be synthesized by their symbionts. Thus, it is important to highlight the possible role of marine bacteria associated with soft coral in providing an alternative to the commercial metal-based antifouling coatings that are believed to be environmental hazards due to their toxicity. Bacteria-seagrass association that occurs on the seagrass surface then could be of great interest to search for potential use as commercial antifoulants.

Here, we report the antifouling activity of bacterial symbionts of seagrass *Enhalus* sp. against marine biofilm-forming bacteria isolated fiber and wooden panels from the surrounding colonies of seagrass *Enhalus* sp.

Materials And Methods

Sampling of seagrass and isolation of bacterial symbionts

Colonies of seagrass *Enhalus* sp. (**Fig. 1**) were collected from sea grass beds in the vicinity of Panjang island, Jepara, Central Java, Indonesia by hands. Upon collection, sea grass colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA. The tissues were then rinsed with sterile seawater and scraped off with a sterile knife. As for endophytes, the surfaces of sea grass were rinsed with 70% alcohol, and the inner parts were cut with a sterile knife. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al.,* 2000).

Isolation of marine biofilm-forming bacteria

Isolation was carried a method modified from Harder *et al.,* (2003). Four pre-sterilized wooden slides had been deployed in 4 different around grass colony for a week. The biofilm developed in these wooden slides were then put into sterile petri dish, rinsed with sterile seawater and scrapped off with a sterile knife. The resultant mixture was diluted. One hundred µl of each dilution was spreaded onto ½ strength ZoBell 2216E and incubated at room temperature for 48 hours. Colonies with distinguished feature were selected and purified.

Antifouling test

Antifouling test of bacterial symbionts of sea grasses against marine biofilm-forming bacteria was performed by using agar diffusion method (Conception *et al.,* 1994). Culture of each marine biofilm-forming bacterium in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread onto Zobell 2216E. Paper discs were then placed on to the respective agar surface. A total of 30 micro litters of bacterial symbionts were poured onto

ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

the paper discs and incubated for 4 d. The plates were then incubated at room temperature for 48 hours. Antibacterial activity was defined by the formation of inhibition zones around the paper discs (Radjasa *et al*., 2007).

Fig 1. Seagrass *Enhalus* sp.

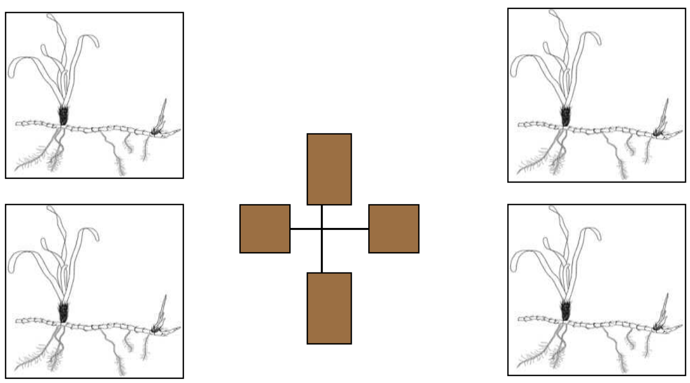

Fig 2. Setting of wooden slides in the surrounding colonies of sea grass

RESULTS AND DISCUSSION

Results

Isolation of marine biofilm-forming bacteria

A total of 23 and 27 isolates were obtained from two types of panel (wooden and fiber), respectively (**Table 1**). It is interesting to note that more isolates were found in both substrates with rough surface compared to smooth surface.

Antifouling test of bacterial epiphytes against marine biofilm-forming bacteria revealed that out of 17 isolates, 8 isolates (47%) were found to be active. On the other hand, among endophytes, all six isolates (100%) were active against the tested biofilm-forming bacteria.

Eight bacterial epiphytes were able to inhibit the growth of biofilm-forming bacteria with a range of inhibition between 0,82 mm – 7,73 mm, with a total of 11 biofilm-forming bacteria were inhibited. On the other hand, 6 bacterial endophytes were all able to inhibit the growth of biofilm-forming bacteria with a

ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

range of inhibition between 1,17mm – 5,8 mm, with a total of 9 biofilm-formfing bacteria were inhibited (**Table 3 and 4**).

Table 1. Number of isolates obtained from wooden and fiber substrates

No	Substrate	Roughness	Number of isolate
1	Wooden panel	Rough	14
		Smooth	9
2	Fiber panel	Rough	15
		Smooth	12

Isolation and antifouling activity of bacterial symbionts

A total of 17 epiphytes and 6 endophytes were isolated seagrass *Enhalus* sp. More epiphytes were found compared to endophytes (**Table 2**).

Discussion

Marine fouling causes huge economic losses to marine industries, since almost all types of structural materials exposed to seawater may become fouled. In seawater the microbial population on surfaces creates an additional problem by producing the primary biofilm, which is generally thought to be a prerequisite for the attachment and metamorphosis of fouling organisms.

Within minutes of immersing a clean surface in seawater, it adsorbs a molecular conditioning film, consisting of dissolved organic material. Bacteria colonize within hours, as may unicellular algae and cyanobacteria. These early small colonizers form a biofilm, an assemblage of attached cells sometimes referred to as microfouling. A macrofouling community may develop and overgrow the microfouling (Callow and Callow, 2002). The most widely used chemical antifoulant TBT, accumulates in the marine sediments and shows negative effect on several marine species. Cooper-based paints are being used as an alternative to to TBT-containing paints, however, the toxicity of copper to species of marine algae and the ability of shellfish to accumulate the metal has increased concerns against the use such paints. TBT-containing paints, however, the toxicity of copper to species of marine algae and the ability of shellfish to accumulate the metal has increased concerns against the use such paints.

The sea grass ecosystem is an important component of coastal ecosystems which provides nursery grounds for relevant fish and invertebrate species, stabilizes sediments and fixes significant of inorganic carbon. However, the health of sea grass communities is sometimes threatened by pathogenics and epibiosis. Therefore, there is ample ecological rationale for sea grass to maintain some types of antimicrobial chemical defense to reduce the rate of infection and surface fouling.

Table 2. Number and activity of epiphytes and endophytes from seagrass

No	Bacterial Epiphyte			Bacterial Endophyte		
	Isolated	Active	Percentage	Isolated	Active	Percentage
1.	17	8	47 %	6	6	100 %

ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

Table.3. Antifouling test of epiphytes of *Enhalus* sp. against biofilm-forming bacteria

NO	Bacterial epiphyte	Biofilm-forming bacteria	Zone of Inhibition (mm)
1.	EEp 1	B.F.1	1,77
2.	EEp.2	B.F.2	0,82
		B.F.3	1,53
		B.F.4	3,77
3.	EEp.3	B.F.5	0,87
		B.F.6	1,38
4.	EEp.4	B.F.7	7,73
5.	EEp.5	B.F.8	1,9
6.	EEp.6	B.F.9	1,97
7.	EEp.7	B.F.10	2,3
8.	EEp.8	B.F.11	1,027

Table.4. Antifouling test of endophytes of *Enhalus* sp. against biofilm-forming bacteria

NO	Bacterial endophyte	Biofilm-forming bacteria	Zone of Inhibition (mm)
1.	EEd.1	B.F.10	1,23
		B.F.12	1,5
		B.F.2	2,23
		B.F.13	1,37
		B.F.10	1,33
		B.F.5	1,17
		B.F.14	2,33
		B.F.15	2,27
2	EEd.2	B.F.2	5,8
		B.F.13	1,67
		B.F.15	5
		B.F.11	2,1
3	EEd.3	B.F.13	1,2
4	EEd.4	B.F.13	5,23
5	EEd.5	B.F.16	3,7
6	EEd.6	B.F.15	4,07

Marine plants and invertebrates are rich sources of biologically active secondary metabolites, many of which provide important antimicrobial chemical defense toward off infection and fouling (Davis et al, 1989). Sea grasses are an important component in the coastal ecosystem along with other productive ecosystems such as coral reefs and mangroves. Sea grasses produce secondary metabolites with ecologically important roles. There is a clear ecological rationale for sea grasses to maintain antimicrobial chemical defense, since

ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

they are susceptible to periodic microbial diseases and shading effects of fouling community that can reduce sea grass photosynthetic rates. Jensen et al (1998) reported that sea grass secondary metabolite regulate associated microbial populations, and thereby provide a type of antimicrobial chemical defense that function via reduced surface fouling.

It has been estimated that less than 2% of microbial flora have been successfully isolated from marine environment as pure cultures. It is expected that still quite a few parts of unexplored culturable sea grass-associated microorganisms exists in the sea grass bed environments. Thus, such information might be desirable, as some of these bacteria may serve beneficial purposes as the source of marine natural products including alternative marine antifoulants.

The results indicated that type of substrates (fiber and wooden panels) greatly affects the diversity of marine biofilm-forming bacteria. The roughness of the substrate also significantly influenced the number of isolates, in which more isolates grew in rough surfaces (14, 15 isolates) than smooth ones (9, 12 isolates). The fact that a rough surface submersed in the seawater provides better surface colonization for the bacterial adhesion. Characklis *et al.,* (1988) mentioned that extended microbial colonization increased in accordance with the roughness of the surfaces. Furthermore, Railkin (2004) reported that the formation of microfouling and macrofouling is partly influenced by the presence of rough surfaces as substrates. Thus, it is reasonable to find more isolates obtained from fiber panel than in wooden panel.

It has been noted that among the active endophytes, 1 isolate was observed to inhibit the growth of 8 isolates of biofilm-forming bacteria and 1 isolate was able to inhibit the growth of 4 biofilm-forming bacteria. In contrast, among epiphytes, 1 isolate was able to inhibit the growth of 3 biofilm-forming bacteria. The present results revealed that endophytes showed stronger ability to inhibit the growth of biofilm-forming bacteria than epiphytes.

All aspects of the biology and interrelatedness of endophytes with their respective hosts is a vastly underinvestigated and exciting field. Thus, more background information on a given plant species and its microorganismal biology would be exceedingly helpful in directing the search for bioactive products. Currently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species.

According to Strobel and Daisy, (2003), two specific rationales for the collection of each plant for endophyte isolation and natural-product discovery that can be used as reasonable strategy are as follows. (i) Plants from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival are seriously considered for study. (ii) Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity.

The present results supported the fact that endophytes from seagrass bed which is widely known as one of the most productive ecosystems in the coastal environment, indeed exhibited microfouling activity, therefore deserve strategic development for the search marine antifoulants.

CONCLUSIONS

Baacterial symbionts (epiphytes and endophytes) of seagrass *Enhalus* sp. showed potential role in controlling the growth of microfouling community, especially marine biofilm-forming bacteria which are thought to play an important role for the development of mature macrofouling community. Endophytes

ISSN : 1410-5217
Accredited : 83/Dikti/Kep/2009

and epiphytes serve as alternative source of environmentally friendly marine antifoulants. The type of substrates: fiber and wooden panels supported different biofilm-forming community, and may influence the growth of different fouling organisms.

ACKNOWLEDGEMENTS

This research was partly funded by a research grant from International Foundation for Science (IFS), Sweden (Contract. No. F/3965-2), awarded to Ocky Karna Radjasa

REFFERENCES

Characklis, W.G. and A.R. Escher. 1988. Microbial Fouling: Initial Event. In Marine Biodeterioration. A.A. Balkema. Rotterdam.

Callow, M.E abd J. A. Callow. 2002. Marine biofouling: A sticky problem. *Biologist.* 49:1-4.

Conception, G.P., Caraan, G.B., Lazaro, J.E. 1994. Biological Assays for Screening of Marine Sample: Work Book Strategies in the Quest for the Novel Bioactive Compound from the Sea. Marine Science University of Philipines, Philipine: 55 pp.

Davis, A., N.M. Targett., O.J. McConnell., and C.M. Young. 1989. Epibiosis of the marine algae and benthic invertebrates: Natural products chemistry and other mechanisms inhibiting settlement and overgrowth. Pp. 85-114. In. P.J. Scheuer (ed). Bioorganic marine chemistry. Springer-Verlag KG. Berlin, Germany.

Jensen, P.R., K.M. Jenkins., D. Porter., and W. Fenical. 1998. Evidence that a new antibiotic flavone glycoside chemically defends the sea grass *Thalassia testudinum* against Zoosporic fungi. *Appl. Environ. Microbiol*. 64:1490-1496.

Madigan MT, Martinko JM, Parker J, Brock TD. 2000. Biology of microorganisms. Prentice-Hall, Inc., New Jersey, USA

Maki, J.S., and R. Mitchell. 1988. Microbial surface film and their influence on larval settlement and metamorphosis in the marine environment. In Marine Biodeterioration: Advanced techniques applicable to the Indian Ocean. A.A. Balkema, Rotterdam.

Railkin, A.I. 2004. Marine Biofouling; Colonization Processes and Defence. CRC Press. Florida.

Smitt, J. 1988. Caulobacters in the marine environment. In Marine Biodeterioration: Advanced techniques applicable to the Indian Ocean. A.A. Balkema, Rotterdam.

Strobel, G., and B.Daisy. 2003. Bioprospecting of microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev*. 67: 491-502.