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Identification of Biofilm-forming Bacteria from Steel Panels Exposed in Sea Waters of Jakarta Bay and Madura Strait

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Abstract. Biofouling formation on marine constructions may cause significant problem such as bridge and vessel damages. Bacterial biofilms are the initiating cause of biofouling. Unfortunately, information of identified biofilm-forming bacteria associated with biofouling process in Indonesian seawaters was still limited. The purpose of this study was to identify biofilm-forming bacteria from biofouling of surface of steel panels exposed in Seawaters of Jakarta Bay and Madura Strait. Steel panels coated either with antifouling or anticorrosion paints were put into sea waters of Jakarta Bay and Madura Strait. Isolation of bacteria was conducted from steel panels fully covered by fouling organisms and on surface without fouling. Confirmation of biofilm-forming activities was performed based on crystal violets-stained bacterial materials on polystyrene well plates. As results, accumulation of macroorganisms and microorganisms was recognized visually on anticorrosion-coated panel that had been put in all observed sea waters for one month. However, biofouling appearances were not recognized on the antifouling-coated panel. Bacterial colonies isolated from Jakarta Bay (108 isolates) and Madura Strait (30 isolates) were screened for biofilm formation on polystyrene well plates. Five isolates representing the most active strain to biofouling activity were then identified based on 16S rRNA gene sequences. As results, isolates of Jakarta Bay C021.MB.1.8, C221.MB.II.11, and C221.MB.II.12 was closely related to *Vibrio alginolyticus* (99 % similarity). Another two isolates of Madura Strait J111.SM and J111.1.10-6.S had similarity to *Vibrio natriegens* (99 % to 100 %). All these biofilm-forming bacteria were isolated from biofouling of panels coated with anticorrosion paints. In addition, we confirmed that *V. alginolyticus* C021.MB.1.8 formed biofilm only if growth medium is seawater from Jakarta Bay. Our study and the bacterial collections will be useful for evaluating and developing new antifouling paints that will be applied in sea waters of these two sites.

Keywords: *Vibrio*, Jakarta Bay, marine biofouling, marine construction, Madura Strait

INTRODUCTION

Constructions and ship hull submerged in seawater are usually colonized by accumulation of microorganisms and macroorganisms. This accumulation of organisms on wetted surfaces is called biofouling. Biofouling development involves the gradual accumulation of organisms such as bacteria, algae, barnacles, and protozoa [1]. The biofouling process can be simplified as follow: formation of conditioning film, absorption of bacteria or microalgae, reorientation and gliding of bacteria or microalgae, secondary adhesion of microfouling organisms, and maturation of biofilm [2]. Bacterial biofilms are therefore the initiating cause of biofouling. Reorientation and gliding of microorganisms is a biochemical reaction where microorganisms secrete extracellular polymeric substances (EPS). In case of Gram-

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negative bacteria, initial attachment is dictated by physicochemical and electrostatic interactions between the surface and the bacterial envelope [3] and occur in a range of molecular sizes, conformations and physical/chemical properties, and polysaccharides, proteins, lipids, and also nucleic acids [4].

This colonization causes serious problems such as bridge and vessel damages. Research from [5] estimated that microbial biofouling is a very costly problem that keeps busy a billion-dollar industry providing biocides, cleaners, and antifouling materials worldwide. Since bacterial adhesion is early stage of biofouling formation and is the fundamental cause of biofouling [1], controlling the bacteria is one good strategy to avoid biofouling development.

Although several investigations on biofouling-associated microorganisms have reported in several areas surrounding Java islands such as Bali [6], Ujung Kulon, West Java [7], and Jepara, Central Java [8], no report has been published in strategic area such as nearby Jakarta and Madura-Surabaya. The purpose of this research was to identify bacteria that are capable to form biofilm from steel panels exposed in seawaters of Jakarta Bay and Madura Strait.

MATERIALS AND METHODS

Steel Panel

This study is in line with research on the corrosivity and performance evaluation of antifouling paint exposed in seawater reported by [9]. Steel panels used in this study were described by [9]. Brievely, steel panels consisted of commercial metal sheet of mild steel (C = 0.13 %, Sil = 0.11 %, P = 0.02 %, Mn = 0.29 %, Ni = 0.01 %, Cr = 0.30 %, Cu = 0.01 %, Ti = 0.01 %, Ti = 0.01 %, Fe = 99 %) with the thickness of 0.3 cm. Sheets of mild steel were cut into required number of specimens of sizes, 20 cm width × 25 cm height for the immersion studies. The specimens were sandblasted according to the standard of ISO 8501-1 and coated by antifouling and anticorrosion paints.

Biofouling Formation and Isolation

The test panels coated by antifouling and anticorrosion paints were exposed up to three months for immersion (depths of 0, 1, 2, 3 meters from sea level) in seawaters of Jakarta Bay, Jakarta and Madura Strait, Surabaya. Biofouling development on steel panels was observed visually. In this study, we conducted the isolation of biofouling bacteria of one month seawater exposure considering the presence of bacteria in a preliminary step of biofouling on steel panels. Any biofouling materials were then scraped from panels and submerged in seawater. Samples were then serially diluted in sterile filtered seawater and plated over seawater agar media that is consisted of 3.4 g peptone (Bacto), 8.5 yeast extract (Himedia), 1 g glucose (Merck), 21 g agar (Himedia) per liter of filtered seawater from Jakarta bay or Madura Strait. The inoculated plates were incubated at room temperature (about 22 °C). After incubation, the selected bacterial colonies were purified and subcultured in seawater agar medium for further investigation according to [10] with some modifications.

Screening of Bacterial Biofilm Formation Activity

Bacterial biofilm formation activity of bacterial colonies was using the crystal violet technique adapted from [10] by using polystyrene 96-well microplate (Iwaki). Bacterial isolates were grown on seawater agar media for 48 h at 37 °C. Each colony was picked up by sterile tip needle (BD Difco™ loops and needles) and dipped into (inoculated) in three parallel wells of a 96 well microtiter plate containing 200 µl seawater media and incubated for 6 d at 37 °C. After the incubation period, the wells were rinsed with physiological saline and fixed with 2 µL of 99.99 % ethanol for 10 min. The attached bacterial material was then stained by adding 2 µL of crystal violet (2 %) for 20 min. The plate was rinsed with tap water gently and the attached biomass was measured using a microplate reader at 570 nm.

Molecular Identification of Biofilm-forming Bacteria

Biofilm-forming bacterial isolates were identified through 16S rRNA gene sequences analysis. Amplification of 16S rDNA sequent was done by colony method of polymerase chain reaction (PCR). Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (provided by Integrated DNA Technologies) were used for amplification of 16S rRNA gene [11]. The purified PCR amplicons were then

sequenced and compared with the 16S rRNA gene sequences available in the GeneBank database at the National Centre for Biotechnology Information (NCBI) (*http://www.ncbi.nlm.nih.gov/BLAST*) on Feb 28, 2018, using the Basic Local Alignment Search Tool (BLAST) program. Clustal X v2.0 software was used for phylogenetic tree construction using the neighbor-joining method with 1 000 time bootstrap [12].

RESULTS AND DISCUSSION

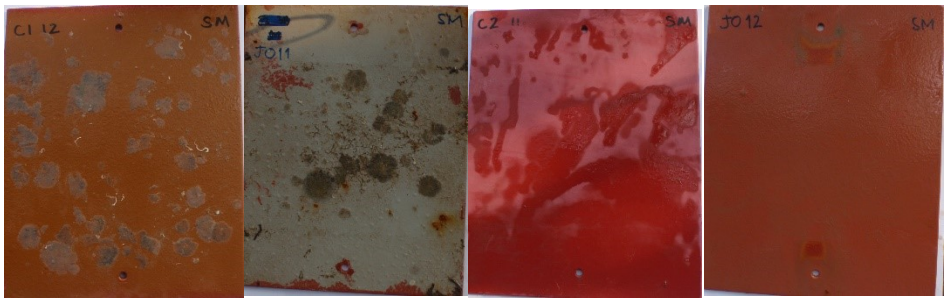
Biofouling Development on Steel Panels

Visual observation of fouling on submerged panels coated with anticorrosion or antifouling paints after 1 month of immersion in seawater of Jakarta Bay or Madura Strait is presented in Fig. 1 or 2, respectively. Biofouling was developed only on panels coated with anticorrosion paints (paint A and B) but not on antifouling paints. Antifouling paint A and B contain copper as main fouling biocide.

Visually, community of fouling organisms developed in seawater of Jakarta Bay was denser than that of Madura Strait. The richness of nutrition in Jakarta Bay seawater may be able to support fouling formation. The hypothesis is supported by the fact that Jakarta Bay receives by far the highest estuarine nutrient load [13].

(a) (b) (c) (d)

FIGURE 1. Biofouling formation on panels coated with anticorrosion A (a), antifouling A (b), anticorrosion B (c) and antifouling B (d) paints after 1 month of immersion in seawater of Jakarta Bay
(The data from the same research has been reported [9])



(a) (b) (c) (d)

FIGURE 2. Biofouling formation on panels coated with anticorrosion A (a), anticorrosion B (b), antifouling A (c), and antifouling B (d) paints after 1 month of immersion in seawater of Madura Strait

Salinity, conductivity, and pH of the two seawaters were practically similar (Table 1). It indicates that different growth of biofouling in these two seawaters is not affected by salinity or pH. Carbon and nutrients, as a prerequisite for biofilm growth, can alter the architecture, community of biofilm [14]. This research did not measure dissolved organic matter however it may consider that the richness of carbon and nutrients could be responsible for the better growth of biofouling in seawater of Jakarta Bay.

TABLE 1. Basic chemical and physical characteristics of seawaters.

Chemical and Physical Characteristics of Seawater	Seawater	
	Jakarta Bay(*)	Madura Strait
Salinity (ppt)	30.3	28.7
Conductivity	48.34	48.27
pH	8.39	8.45

(*) The data from the same research has been reported [9]

(a) (b) (c) (d)

FIGURE 3. Biofilm formation on panels coated with antifouling A after three months of immersion (a), without immersion (b), antifouling B after 3 months of immersion (c), and without immersion (d) in Jakarta Bay seawater

Biofilm development on panels coated with antifouling paints was also elaborated (Fig. 3). On panel coated with antifouling immersed in seawater of Jakarta Bay, dense crystal violet was visually observed but not on the panel before immersion (control). Organic materials that stuck on the panel may be revealed by crystal violet. The data indicated the very early stage of fouling (organic materials adhesion) [2] might happen. However, the formation of biofilm on panel coated with paint B was difficult to interpret since control panel also adsorbs crystal violet.

Screening of Biofouling Bacterial Isolates

This research successfully isolated 108 different bacterial colonies from organism communities of Jakarta Bay and 30 isolates from Madura Strait (data not shown). Biofouling activity of isolates with highest biofilm-forming activity was presented in Fig. 4.

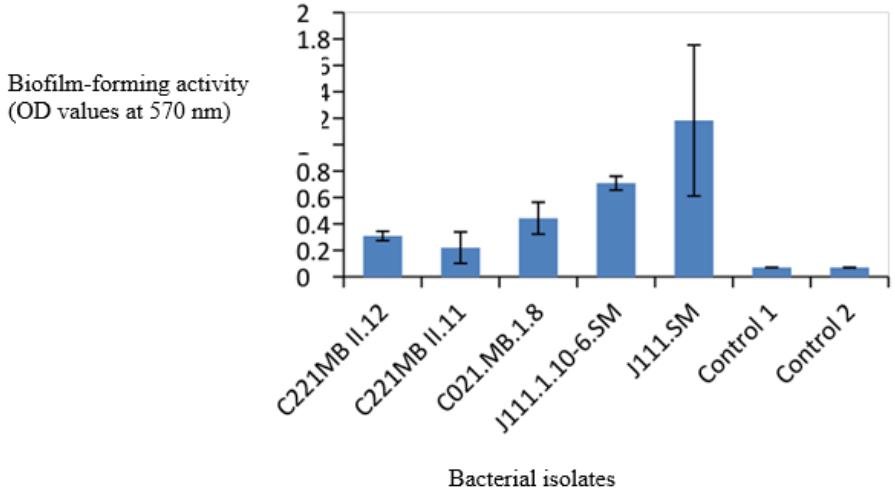


FIGURE 4. Activity of biofilm-forming

Isolates C021.MB.1.8, C221.MB.II.11, C221.MB.II.12 (from Jakarta Bay), J111.1.10-6.SM, J111.SM (from Madura Strait) were active to form biofouling (Fig. 4). Thus, these five biofouling-associated bacterial isolates were

identified at the species level. The data showed that some other bacterial isolates associated with biofouling are not capable of forming a biofilm. However, their role in biofouling development could not be neglected. Research by [15] showed that the interaction among an initial biofilm-forming bacteria and subsequent colonizing biota and abiotic factors is important in the developing biofouling community, although its mechanism of action is still not well understood.

Molecular Identification of Biofouling Bacterial Isolates

Molecular identification showed that isolates of Jakarta Bay C021.MB.1.8, C221.MB.II.11, and C221.MB.II.12 had closest relationship to *Vibrio alginolyticus* (99 % similarity). Two isolates of Madura strait J111.1.SM and J111.1.10-6.SM were identified as *Vibrio natriegens* with the similarity of 99 % to 100 % similarity (Table 2). Those isolates were clustered convincingly in two group of *Vibrio alginolyticus* and *Vibrio natriegens.* Although all isolates from Jakarta bay were related to *V. alginolyticus,* the nucleotide sequence of 16S rRNA gene of C021.MB.1.8 is slightly different as compared to C221.MB.II.11 and C221.MB.II.12 (Fig. 5) was found. Species *V. alginolyticus* and *V. natriegens* had similarity in nucleotide sequence of several genes. The phylogenetic tree based on the neighbor-joining method using the 16S rRNA, recA and rpoA c confirmed that *V. alginolyticus* and *V. natriegens* are clustered into one group as well other *Vibrio* species such as *Vibrio parahaemolyticus, Vibrio diabolicus, Vibrio harveyi,* and *Vibrio campbellii* [16] [17].

TABLE 2. BLAST results of 16S rRNA sequences of biofouling bacterial isolates obtained from seawaters of Jakarta Bay and Madura Strait.

Source of seawater	Isolate	Closest relationship	Accession No.	Similarity (%)
Jakarta Bay	C021.MB.1.8	*Vibrio alginolyticus* NBRC 15630	NR113781.1	99
Jakarta Bay	C221.MB.II.12	*Vibrio alginolyticus* NBRC 15630	NR122050.1	99
Jakarta Bay	C221.MB.II.11	*Vibrio alginolyticus* NBRC 15630	NR122050.1	99
Madura Strait	J111.1.SM	*Vibrio natriegens* ATCC 14048	NR117890.1	100
Madura Strait	J111.1.10-6.SM	*Vibrio natriegens* ATCC 14048	NR117890.1	99

Genus *Vibrio* was recognized as an important bacteria group that is responsible for marine biofouling [18]. Bacteria involving in biofouling from several marine environments have been previously studied. In preliminary study of biofouling-associated bacteria, several strains capable of forming biofilm were collected in glass slide after dipping in seawater surrounding soft coral *Sarcophyton* sp. colony for a week in Peucang Island, Ujung Kulon, West Java, Indonesia [7]. Biofilm-forming bacteria were also successfully isolated from colony of seagrass in Teluk Awur waters, Jepara, Central Java, Indonesia [8]. Those unidentified biofilm-forming strains were then applied for screening antifouling microbes isolated from soft corals. Biofouling-associated bacteria isolated from Segara Ayu Beach, Bali, Indonesia have been identified as *V. alginolyticus, V. natriegens, V. neocaledonicus, Pseudomonas stutzeri,* and *Shewanella* [6]. In corresponding to this study, *V. alginolyticus* and *V. natriegens* were commonly found in seawaters, especially marine environment surrounding Java islands.

Bacterial biofilm was related substances excreted extracellularly such as polysaccharides. Marine bacteria *V. alginolyticus* was reported to produce exopolysaccharide (EPS) production in seawater nutrient broth in vitro. The EPS of *V. alginolyticus* had molecular weight about 6 390 kDa with good shearing property [19]. Another species *V. natriegens* reported in this study has not been intensively investigated as biofouling organism. However marine *V. natriegens* was reported to play a role in the microbial corrosion of stainless steel as determined by electrochemical techniques and surface analysis [20]. Other species in the genera of *Vibrio* were also reported in the production of extracellular polymeric substances such as marine bacterium *V. parahaemolyticus* [21]. *V. harveyi* [22], and *V. fischeri* [23].

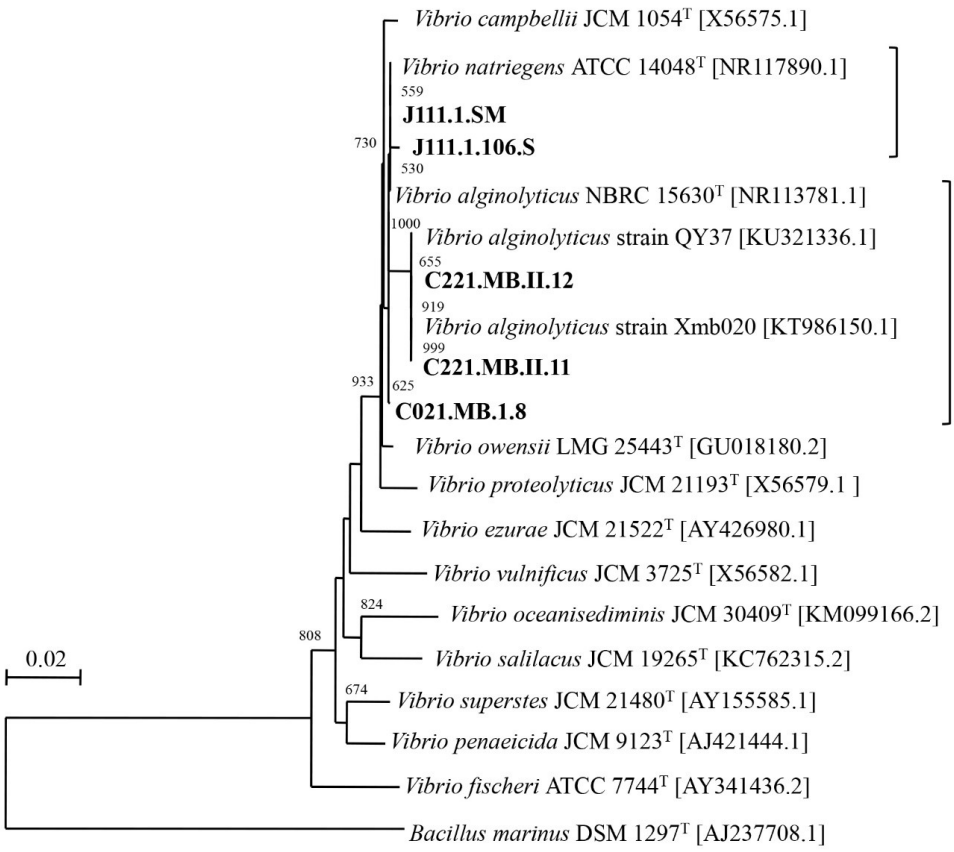


FIGURE 5. Phylogenetic tree of bacterial isolates associated with biofouling representing *Vibrio* spp. based on 16S rRNA gene sequencing by neighbor-joining method. Distances were estimated according to the Kimura two-parameter model with bootstrap percentages after 1 000 simulations. The bar represents a 2 % estimated sequence divergence. *Bacillus marinus* DSM 1297^T was used as an outgroup

Effect of Seawater Source on Biofouling Activity

In order to understand the possibility of nutritional factor that could affect the activity of biofouling, this study used three different seawater media to confirm their effect on biofouling activity of isolate from Jakarta Bay *V. alginolyticus* C021.MB.1.8. The result was presented in Table 3.

TABLE 3. Effect of seawater source on fouling of *V. alginolyticus* C021.MB.1.8.

Seawater	Fouling formation
Madura Strait	-
Jakarta Bay	+
Synthetic (Difco Marine Broth)	-

- : inactive ; + : active

Table 3 showed that isolate from Jakarta Bay *V. alginolyticus* C021.MB.1.8 formed fouling layer on polystyrene microplate only when growth medium is seawater of Jakarta Bay. This data indicated the possibility of nutritional factor growth media content that affects biofouling development.

CONCLUSION

This study showed that fouling attachments to steel panels coated with anticorrosion paints submerged in seawaters of Jakarta Bay and Madura Strait were observed after a month of immersion. Different species from the genus *Vibrio* identified as *V. alginolyticus* and *V. natriegens* were responsible for biofouling. Biological and chemical components of seawaters might affect settlement pattern of bacterial biofoulers.

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