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Research Article
Exopolysaccharides-Producing Biofilm Bacteria from Submerged Seawater Substrate for Bioremediation of Heavy Metal Contamination

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Abstract

Background and Objective: The coastal environment is often polluted by various toxic compounds such as heavy metals. Exposure to these toxic compounds causes coastal bacteria to adapt so that they can be used as bioremediation agents for heavy metals. This study aims for finding and screening the ability of bacteria to produce exopolysaccharide biofilms and then determine the characteristics of bacterial isolates as agents candidates for heavy metal bioremediation in the coastal environment. **Materials and Methods:** Samples were collected on submerged seawater substrate from Bungus Coastal, Padang and West Sumatra, on the wet area that was exposed by seawater (on the rocks, on the wood and the ship, the lower out part on the ship that exposed to seawater). Bacterial isolation process using Marine Agar Medium. The isolate discovered then observed and purified. Furthermore, Congo Red Agar was used for bacteria screening for detecting EPS produced by biofilm bacteria. **Results:** The results of the isolation, found 9 bacterial isolates attached to the substrate submerged seawater. The screening results showed that isolates K4, K5 and K7 were positive as biofilm-forming bacteria as indicated by the colour change of the bacterial colonies to black on Congo Red Media after 24 hrs incubation. The characteristics of the three bacterial isolates were gram-negative, with cocci and bacilli cells form. **Conclusion:** Three isolates of positive exopolysaccharide biofilm bacteria that 1 isolate gram-negative coccus (K4) and the other 2 isolates (K5 and K7) were bacillus. Then, the 3 isolates can be used for remediation of metal contamination research in aquatic.

Key words: Bacteria, biofilm, bioremediation, Congo Red Agar, exopolysaccharides, heavy metal, marine

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The marine environment is the biggest aquatic ecosystem on earth and has harboured microorganisms that are responsible for more than 50% of the world's total prokaryotic biomass. All the microorganisms produce extracellular polymers that constitute a substantial part of the dissolved organic carbon, often in the form of Exopolysaccharides (EPS). In addition, this production of polymers is often correlated with the formation of biofilm growth mode, where they are an important component of the matrix. Their functions include adhesion and colonization of surfaces, bacterial cell protection and support for the biochemical interactions between bacteria and bacteria surroundings[1].

Marine bacteria are highly adaptive to contaminated environments. Because their constant exposure to this contaminant such as heavy metals make them become the best utilization for bioremediating heavy metals. Therefore monitoring and assessment of marine microbial resistance can be powerful tools in bioremediation. This aspect of heavy metals resistance could be utilized for pollutants removal from contaminated environments. Activity by the marine bacteria has been recognized as one of the most significant and efficient approaches for removing toxic metals from the environment[2].

Bioabsorption of heavy metals has gained much attention in recent times because of the potential use of microorganisms for cleaning the environment that is contaminated with heavy metals pollutants. Biofilm bacteria have been proved to be more favorable in decrease the pollutant in terms of efficiency and easier processing than the planktonic bacteria. The biofilm bacteria can be useful for removing the toxic metals from effluent and covering metals from diluted solution[3]. At the sea surface, biofilms can arise as slicks, such as part of the sea surface slick and slimy than the sea surface around them. Biofilms are usually defined by the accumulation of excessive particles and microbes. In some studies assessing the presence of potential distribution and the frequency of the formation of a slippery area in coastal and marine areas, can provide influence on the air-sea CO_2 exchange. It is estimated that slicks can reduce CO_2 flux by up to 15%[4].

Biofilm and the marine microbes are still largely unexplored and exploited, Therefore, it is necessary to know more about the biofilm and their bacteria also the EPS produced by marine microbes that can be used to identify the biofilm, to manage them become less damaged or even better, being useful in industrial or on the everyday product. Biofilm also becomes a promising bio-agent to treat heavy metal pollution especially in natural source water and wastewater treatment processes.

MATERIALS AND METHODS

Study area: The study was carried out at the Microbiology Laboratory of the Department of Biology Andalas University from April-October, 2020. Sampling was carried out on submerged seawater substrate the Bungus Coastal, Padang and West Sumatra. Biofilm was scraped off from their substrate parts of the Coastal that were on the wet area that was exposed by seawater.

Bacteria isolation: Sampling was done by taking parts of biofilm at Coastal of Bungus which allegedly contained biofilms. Parts of the coastal that were on the wet area that was exposed by seawater. There was 3 sampling area that collected, they were on the rocks, on the wood and the ship, the lower out part on the ship that exposed to seawater. The biofilm with slime texture was then scrapped, keeping in a sampling bottle and saved in a cooler box. Samples then moved into a laboratory for further work. The samples of biofilm were diluted and then inoculated in Marine Agar Medium with the pour plate technique. Samples that have been inoculated are then incubated for 24 hrs[5]. After that, the bacteria colony was observed and then purified. Macroscopic observations were made by directly observing the forms, elevation and margins of bacterial colonies[5].

Screening of biofilm bacteria: To found the biofilm isolate bacteria by using Congo Red Agar as a medium to observe the bacteria that producing EPS. Testing the EPS-producing bacteria was observed by growing the isolated bacteria on Congo Red Agar medium. The bacteria were scratch and incubated with a medium Congo Red Solid. Bacteria were incubated at 37°C for 24-48 hrs in aerobic[6]. Bacteria produced exopolysaccharides identified as biofilm bacteria will form a black coloured colony with a black coloured concentration of dry crystals. Whereas a negative result is non-biofilm bacteria will form colonies of red or pink[7].

Microscopic observations and biochemical tests: Microscopic observation was carried out with gram staining to determine whether the bacterial isolate was gram-positive or gram-negative and to determine the shape of the bacterial cell. Biochemical tests will be carried out based on the results

of gram staining and cell shape. If the bacterial isolate obtained is gram-positive and in the form of bacilli, a spore staining will be carried out, while in the form of coccus the catalase will be tested. If the bacterial isolate obtained is gram-negative and coccus-shaped, a glucose test will be carried out, while the bacillus form will be a lactose test[5].

RESULTS AND DISCUSSION

The data in Table 1 shows the results of the biofilm screening test with congo red agar. Three of them were positive for exopolysaccharide biofilm bacteria from nine bacteria tested since the colonies showed black colour on the colonies after incubation for 24 hrs. The black colour of the colonies becomes more intense within 48 hrs. However, the other 6 isolates showed negative biofilms because the colonies remained reddish-pink after incubation for 24-48 hrs. It's possible to discover negative biofilm isolates from substrate samples submerged in seawater since these bacteria obtained a better environment in the biofilm matrix, because the EPS biofilm formed a layer around the cells, therefore it provides effective protection against high or low temperature and salinity, or possible predators. Biofilms also supply nutrients for bacteria, such as carbohydrates and proteins[8].

In previous studies, biofilms have also been found on the rust layer of ship steel submerged in seawater naturally, which include colonies of sulfate-reducing bacteria *D. caledoniensis* and iron-reducing bacteria *Clostridium* sp.[9]. To determine the isolates of EPS-producing bacteria, screening was carried out biofilm bacteria. The strong biofilm-forming bacteria showed black crystals in the colonies indicating a positive biofilm, while the pink to reddish-negative bacterial colonies were classified as negative biofilms (Fig. 1a-b). Screening of biofilm-forming bacteria using a specific medium Congo Red Agar after 24 hrs of incubation showed differences in the colour of the colonies growing on the medium. Black colonies indicated that the bacterial isolates were positive for Exopolysaccharide biofilm establishment, while reddish-pink colonies were negative for biofilm establishment[10].

The isolation of coastal bacteria that found in Bungus, parts of the coastal on the wet area that exposed by seawater are using Marine Agar Medium. Marine Agar is a medium that is used to obtain marine bacteria. Marine Agar is a nutrient agar that contains peptone and yeast extract, which also consists of minerals imitated seawater minerals. Marine Agar is common media that is used as a medium for identified marine bacteria[11]. Marine bacteria have potential and are

Table 1: Biofilm assay result from bacteria isolated from Bungus submerged seawater substrate

Isolate codes	Biofilm test
K1	Negative
K2	Negative
K4	Positive
K5	Positive
K6	Negative
K7	Positive
K8	Negative
K9	Negative
K10	Negative

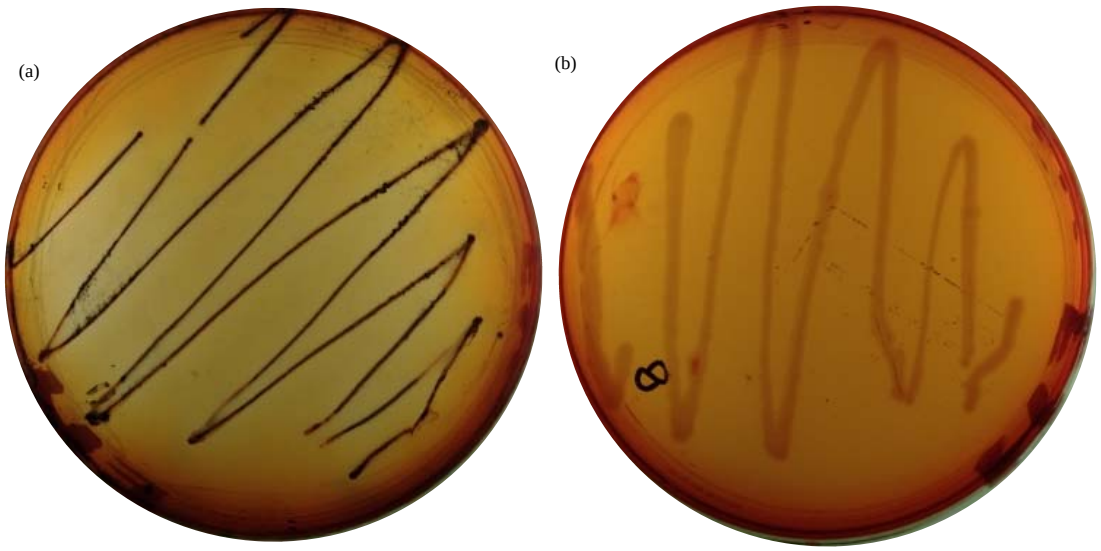

Fig. 1(a-b): Biofilm assay using congo red agar, (a) Positive biofilm shows the colour change in the bacteria colony, becoming black colony and (b) Negative biofilm didn't show a change in the colour colony, the colony remain in red colour

Table 2: Macroscopic observation, microscopic observation and biochemical test of positive biofilm isolated from Bungus submerged seawater substrate

Isolate codes	Macroscopic observation				Microscopic observation		Biochemical test	
	Colors	Shape	Margin	Elevation	Gram stain	Cell shape	Lactose	Glucose
K4	Yellowish	Circular	Entire	Raised	Negative	Coccus	-	Negative
K5	White	Circular	Entire	Raised	Negative	Basil	Positive	-
K7	White	Irregular	Lobate	Flat	Negative	Basil	Negative	-

-: Not tested

highly efficient especially in adapting to a contaminated natural environment. In advance, monitoring and assessment of their microbial resistance can be powerful in discover bioremediation tools[2].

The colony from isolation was then purified on Marine Agar Medium by using the streak plate technique. From the direct observation on bacteria isolation by observed the macroscopic character, there are nine different isolates are found in biofilm from the Bungus coastal area. Those also are determined by their different macroscopic appearances, such as the shape, colour, margin and elevation of the colony. From the nine isolates, the possibility there are EPS-producing biofilm bacteria founded because the samples are biofilm in submerged seawater substrate. The biofilm sample was collected in the rock and wood surface and also part of the ship that has direct contact with seawater. Biofilm can become a bioremediation agent, act as water purification in the natural environment and other contaminants from flowing waters. Biofilm for purifying water from municipal or industrial sources are long have been exploited by collaborated the biofilm with current water purification systems design, including tricking filters in wastewater treatment and point-use device technology[12].

The composition of the biofilm matrix generally consists of carbohydrates, proteins, lipids and nucleic. Carbohydrates are the main component. The chemical components of carbohydrates and molecular weights may differ in the biofilm matrix and cell surface[13]. EPS can also be found covalently bound to the cell surface in the form of a capsule, or present on the cell surface in the form of mucus. EPS-producing bacteria are also found in extreme marine environments such as at high temperatures and pressures and in heavy metal content[14].

Bacteria can survive in environments contaminated with heavy metals such as iron, since they have genes that code metal resistance, because of the process or adaptation mechanism to environmental conditions. Bacterial biofilm can be used for bioremediation in reducing metal contamination. The bioremediation process of heavy metal polluted waters can apply bacterial biofilm exopolysaccharides in a continuous reactor as a practical application[15]. Biofilm isolates can tolerate heavy metals such as mercury, lead, chromium, nickel, zinc and arsenic. Analysis of cadmium reduction by Exopolysaccharide biofilm bacteria showed the best decrease percentage[16]. The application of bacterial exopolysaccharide biofilms is promising for cadmium remediation in waters, due to its better absorption capacity[12].

Metal pollution in waters can threaten human health and ecosystems. Accumulation of heavy metals due to anthropogenic activities causes toxicological manifestations. Safe, effective and environmentally friendly methods of biological remediation are necessary. The existence of microbial groups in the environment contaminated with heavy metals makes these microbes to be able to tolerate these heavy metals. Production Exopolysaccharide (EPS) is one of the microbial strategies to survive on metal pressure so that it can be used as a metal contamination bioremediation agent[17].

The data in Table 2 shows the macroscopic and microscopic characters of the three positive isolates. Three positive isolates of exopolysaccharide biofilms were characterized by different colours and colony shapes. Gram staining showed that isolate K4 was gram-negative, cells were cocci-shaped and negative for a glucose test, while K5 was gram-negative, bacillus cell shape, positive for lactose test and K7 isolate was gram-negative, cells were bacillus, negative for lactose. This difference in character is proof that the three exopolysaccharide-positive bacterial isolates are different from each other. For further evidence, molecular identification of the three bacteria is required.

In addition to bioremediation, biofilm is also a promising technology to increase aquaculture production such as improving water quality, nutrient availability, survival rate and fish growth[18]. Exopolysaccharides are also getting attention as cancer drugs. The current treatment causes side effects and multi-drug resistance. Sulfur-containing polysaccharides and uronic acid exhibit antioxidant activity, by restoring cell redox regulation, therefore inhibiting cancer cell proliferation[19]. In addition, exopolysaccharide bacteria are also used as raw materials for various commercial applications in the fields of chemicals, textiles and cosmetics[20]. In the pharmaceutical field, exopolysaccharide biofilm bacteria have the prospect of

immunomodulatory and antiviral effects, bone regeneration and cicatricial capacity. The food processing industry is also used for its gelling and thickening properties. Meanwhile, in the environmental field, it is important for the detoxification mechanism of biosurfactants and waters contaminated with petrochemical oils[21]. The three isolates that positive exopolysaccharide biofilm bacteria can be used for remediation of metal contamination research in aquatic but this study need to be complete with continuous research to optimize the ability of bacterias found.

CONCLUSION

There are 9 bacterial isolates were founded and successfully isolated from the substrate submerged in seawater in the Bungus coastal area. Three of the isolates were positive for exopolysaccharide biofilm with the character of the three isolates are gram-negative. Isolates cell shaped of K4 is coccus and negative for a glucose test. Isolate K5 negative for lactose test and K7 positive for lactose test. Both isolates K5 and K7 were bacillus forms.

SIGNIFICANCE STATEMENT

This research has found three bacterial isolates of biofilm exopolysaccharides, which will then appear in several further studies related to their potential and utilization in bacterial biofilm technology for bioremediation of heavy metal pollution, especially in the aquatic environment.

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