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Isolation and identification of Rhizobacteria in *Marsilea crenata* Presl. exposed to linear alkylbenzene sulfonate detergent

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ABSTRACT

Water clover (*Marsilea crenata* Presl.) is a plant able to grow well in wetlands contaminated with Linear Alkylbenzene Sulfonate (LAS) detergent. The ability *M. crenata* to absorb this detergent was found to be often assisted by bacteria at the root through phytoremediation process. The aim of the study was to isolate and identify bacteria in the roots of *M. crenata* exposed to LAS detergent. Samples of M. *crenata* roots exposed to 20 ppm LAS detergent were taken, lysed, and homogenized. After that, dilution was performed on the homogenate, before it was grown on NA medium. The different isolates that were grown were purified in a test tube containing NA medium to obtain pure isolates. Each isolate was identified based on macroscopic and microscopic characteristics in addition to physiological characteristics through biochemical tests using the Microbact Identification System Kit. Content of LAS in plant media were analyzed using spectrophotometry. Morphology results were identified using Bergey's Manual of Determinative Bacteriology. The data obtained were analyzed descriptively qualitatively. Decreasing in LAS content after 10 days of treatment was found in the plant, while results of bacteria identification obtained four species of rhizosphere microbes found in the roots that previously known to have a role in LAS phytoremediation; *Bacillus myocides* (similarity coefficient of 80%), *Actinomyces odontolyticus*(90%), *Bacillus pantothenticus* (70%), and *Actinomyces viscosus* (90%).

Key words: *Marsilea crenata, Bioremediation, Detergent, Bacteria, Roots*

Introduction

Detergent waste is one of the largest liquid organic wastes that causes pollution in waters comes from the results of human activities. Detergent waste possibly comes from by-products of domestic, industry, laundry services, and restaurant activities. The residual results of used detergent entering the water body can reduce the quality of the waters and affect the life of the aquatic biota. The detergent waste produced by various activities tend to exceed the threshold set by the Decree Governor of East Java No. 72 of 2013, at maximum LAS level of 10 mg/l.

Water pollution is mainly caused by the active ingredients of detergents, including Sodium Tripolyphosphate (STPP) and additives such as bleaching agents, fragrances, and softeners. These chemicals are difficult to be degraded naturally. In addition, water pollution is also caused by anionic surfactants, such as Linear alkyl Benzene sulphonate

(LAS). LAS is an anionic surfactant composed of a benzene ring and sulfate group, which confers its hydrophilicity, along with an alkyl chain of 10 to 14 carbon atoms arranged linearly, responsible for its hydrophobic property (Hampel *et al.*, 2008, Barra Caracciolo *et al.*, 2017) Detergents containing LAS have the ability to foam 10-13% of its active organic and polyphosphate ingredients, which subsequently produce waste containing phosphorus. The phosphorus content can cause eutrophication, subsequently cause harm and death of aquatic organisms and human (Asok and Jisha, 2012). The phosphate content in detergents can fertilize aquatic weeds and algae to grow beyond normal limit (blooming), reducing dissolved oxygen content and decreasing water quality. For humans, it can cause skin and eye irritation, as well as damage to internal organs, such as kidneys and bile if the chemicals enter the body. Thus, it is necessary to reduce the LAS levelin the waters. One of the methods can be used is phytoremediation.

Phytoremediation is a technique to minimize or reduce pollutants by utilizing plants and theirparts that cooperate with microorganisms in a reactor or directly in a field (Saini *et al.*, 2021). The phytoremediation method involves a root system, as plant roots have the ability to translocate, bioaccumulate, and degrade pollutants. In general, aquatic plants are used as water phytoremediators because they have higher growth rate and ability to absorb contaminants such as detergents faster, for example *Sagitaria lancifolia* (Fitrihidajati *et al.*, 2020), *Pistia stratiotes* which at density of 35 mg/cm^2 was able reduce phosphate by 99%, BOD of 98%, COD of 96% (Raissa and Tanghau, 2017), *Ludwigia adscendens* L. and *M. crenata* (Rachmadiarti *et al.*, 2020). In the phytoremediation process, plants, including *M. crenata*, are assisted by bacteria in the roots or phytoremediation using rhizofiltration techniques (Ali *et al.*, 2013; Singh, 2012; Rajkumar *et al.*, 2012). The use of rhizofiltration in phytoremediation is more beneficial because there is a link between microbes and phytoremediator plants. Phytoremediator plants produce root exudates which can be used by microbes as energy source, while plants benefit in terms of reducing the toxicity of organic and inorganic wastes that will be absorbed (Rajkumar *et al.*, 2012).

The relationship between phytoremediator plants and rhizosphere bacteria in the rhizosphere is the basis for the development of research on bacteria that play a role in reducing levels of heavy metals in the environment, for example *Pseudomonas aeruginosa*, *Streptomyces Tendae*, *Burkholderia caribensis*, *Bacillus sp.*, *Pantoea sp.*, and *Enterobacter sp.* (Park *et al.*, 2011; Wani *et al.*, 2007; Delvasto *et al.*, 2009; Dimkpa *et al.*, 2009). Based on the background that has been described, this study aimed to isolate and identify bacteria species found in the rhizosphere of the roots of *Marsilea crenata*. This plant has been known as phytoremediator plant (Rachmadiarti *et al.*, 2019; Rachmadiarti *et al.*, 2020).

Materials and Methods

Materials

The materials used in this study were samples of *M. crenata* roots taken from the treatment of *M. crenata* exposed to 20 ppm LAS detergent solution, NA (Nutrient Agar) media, sterile distilled water, and sterile physiological solutions. Gram staining ingredients consisted of: Crystal violet, iodine, 95% ethyl alcohol, and Safranin. Materials for endospore staining consisted of: Malachite green and safranin. As well as other materials, such as rubbing alcohol, aluminum foil, cotton, label paper, tissue.

Analysis of LAS content

LAS levels in plant media were analyzed using UV-Vis spectrophotometer at 652 nm.

Isolation of bacteria

Isolation of rhizobacteria was carried out by taking and lysing 10 grams of root samples from the experimental results of 20 ppm LAS detergent exposure, and added it into 90 ml of sterile distilled water. Then mixture was homogenized using shaker for 15-20 minutes. After that, dilution was performed up to 106 times using sterile distilled water. Inoculation was carried out using pour plate method, started by adding 1 ml of the solution at dilutions to 104, 105, and 106 into different sterile petri dishes. Sterile NA medium was then poured into petri dish and incubated at 30 °C. Each different bacterial colony grown was purified on a test tube containing oblique NA medium.

Morphological Observations

Pure isolate was inoculated back in the petri dish containing NA media and incubated for 24 hours at 30 °C. The macroscopic character of the growing

bacterial colonies was observed. Observations of macroscopic characters include colony shape, surface, colony color, edges, and elevation. In addition, microscopic observations were also carried out using Gram stain and endospore staining to determine cell shape, Gram type, and the presence of endospore.

Physiological Test

Physiological tests were carried out using Microbact Identification System Kits (Microbact TM GNB 12A and 12B). This test was used to determine the physiological characteristics of respective bacteria colonies.

Identification of Bacteria

From the results of macroscopic, microscopic, and physiological observations, bacterial isolates were lastly identified of its species using Bergey's Manual of Determinative Bacteriology Eighth Edition (Holt, JG, 1994, William and Wilkins Baltimore).

Results and Discussion

Before isolating and identifying bacteria, *M. crenata* analyzed was exposed first with LAS detergent solution at 10 ppm, 20 ppm, 30 ppm. Results of LAS detergent level after 10 days of treatment are presented in Table 1.

The ability of roots to concentrate detergent LAS is related to the function of plant roots to adsorb on the root surface, precipitate pollutant precipitates, and accumulate in the root zone resulting in LAS ions being absorbed into the roots. The process is then followed by rhizodegradation stimulation of decomposition by microorganism activity by releasing products into the root zone (Zhang *et al.*, 2010). In this study, rhizosphere microbes thought to play a role in degrading LAS has been identified.

Result showed that there were four bacterial isolates with two different genera found in*M. crenata* roots. The results of macroscopic characteristics in the form of colony morphology is presented in Table 2. Bacterial isolates code 5.5 KM Cre and 6.4 KM Cre have similarities in shape, edge, and colony color that are circular, entire, and cream, respectively. The bacterial isolates code 6.4 M Cre and 7.4 M Cre have similarities in the elevation and surface of the colony that are flat and rough. Differences in colony morphology in bacteria can be caused by environmental and genetic factors. Environmental factors can cause temporary or permanent changes in colony morphology and bacterial physiology (Melliawati, 2009).

Observation of microscopic characteristics in the form of cell shape and Gram type were obtained through Gram staining. The presence of endospores was known through endospore staining with Malacite Green staining. The result of microscopic characteristics identification is presented in Table 3. The four bacterial isolates obtained had the same rods cell shape and stained Gram positive. The shape of bacteria cells depends on genetic traits. Bacteria with rod cell shape is known to be able to reduce LAS in polluted environments, for example genus *Bacillus* (Afzal *et al.*, 2014; Shin *et al.*, 2012). The difference is the ability to form endospores. Bacterial isolates with code 5.5 KM Cre and 6.4 KM Cre can form endospores, while bacterial isolates code 6.4 M Cre and 7. 4 M Cre can't form endospore. Endospores are a form of self-defense in unfavorable

Table 1. Detergent LAS levels in the growing media after 10 days of treatment

Plant Species	LAS levels in planting media with LAS media concentration (ppm)			
	0	10	20	30
M. crenata	0.00 ± 0.00	1.84 ± 0.25	2.64 ± 0.25	3.51 ± 0.42

Table 2. Observation result of macroscopic characteristic

No.	Macroscopic Character	5.5 KM Cre	6.4 KM Cre	6.4 M Cre	7.4 M Cre
1	Shape of colony	circular	circular	punctiform	irregular
2	Elevation	convex	flat	flat	flat
3	Edge	entire	entire	entire	undulate
4	Colour	cream	cream	cream	transparent
5	Surface	smooth	Rough	Rough	Rough

Table 3. Observation result of microscopic characteristics

No.	Character	5.5 KM Cre	6.4 KM Cre	6.4 M Cre	7.4 M Cre
1	Gram	positive	positive	positive	positive
2	Cell shape	stem	stem	stem	stem
3	Endospores	+	+	-	-

conditions that are owned by bacteria.

The results of biochemical tests performed using Microbact Identification System Kit is presented in Table 4. Based on these results the four bacteria showed positive catalase. Positive catalase indicates that these four bacteria can produce the enzyme catalase or peroxidase (H_2O_2). Peroxidase has toxic properties because it can damage for cell components. These results involve changing the reaction from peroxidase to H_2O and O2 (Ulfa *et al.*, 2016). The presence of O_2 produced can play a role in increasing dissolve oxygen in the waters so as to improve water quality. The results of the oxidase of the four bacteria showed positive oxidase. This shows that the four types of bacteria need oxygen for their metabolic processes.

The same physiological test results from the four types of bacteria were the negative production of indole. Indole is formed from tryptophan metabolism and is an alkaloid type (Hazid and Zainul, 2005). One type of indole is IAA (Indole Acetic Acid). IAA (Indole Acetic Acid) is a hormone that is widely distributed in the bacterial-plant association system. IAA can indirectly increase metal accumulation by increasing plant biomass (Schalk *et al.*, 2011; Babu *et al.*, 2013). This shows that the four types of bacteria have another mechanism to deal with heavy metal stress (Kuffner *et al.*, 2001), inor-

Table 4. The biochemical and physiological test of isolate bacteria.

No.	Character	5.5 KM Cre	6.4 KM Cre	6.4 M Cre	7.4 M Cre
1	Oxidase	+	+	+	+
2	Motility	-	-	-	-
3	Nitrate	+	-	+	+
4	Lysine	+	-	+	+
5	Ornithine	-	-	-	-
6	H2S	-	-	-	-
7	Glucose	+	+	-	-
8	Mannitol	+	+	-	-
9	Xylose	+	-	-	-
10	ONPG	-	-	-	-
11	Indole	-	-	-	-
12	Urease	-	-	-	-
13	VP	-	-	-	-
14	Citrate	+	-	+	+
15	TDA	-	-	-	-
16	Gelatin	+	+	-	-
17	Malonate	+	-	-	-
18	Inositol	+	-	-	+
19	Sorbitol	+	-	-	+
20	Rhamnose	-	-	-	+
21	Sucrose	+	-	-	+
22	Lactose	+	-	-	-
23	Arabinose	+	-	-	+
24	Adonitol	-		-	-
25	Raffinose	+	-	-	+
26	Copy	+	-	-	-
27	Arginine	+	+	+	+
28	Catalase	+	+	+	+

ganic and organic pollutants, including phosphates (Halman, 2001). One of such mechanisms is the formation of siderophores by *Actinobacillus sp.* on heavy metal stress (Kuffner *et al.*, 2010).

The results of glucose fermentation in bacterial isolates of code 5.5 KM Cre and 6.4 KM Cre showed positive results. In the metabolic process, glucose as an energy source (carbon source) is converted into Adenosine Tri-Phosphate (ATP). During the process of resistance to LAS, ATP plays a role as the main energy source in the efflux system process (Anna and Zofia, 2014). However, bacterial isolates with the code 6.4 M Cre and 7.4 M Cre showed negative results on glucose fermentation. In addition to glucose, the results of sucrose fermentation on bacterial isolates of code 5.5 KM and 7.4 M Cre were positive. This shows that these two bacteria are able to ferment several kinds of sugars in their metabolic process.

These results indicate that there are four bacterial isolates from *M. crenata* roots. 5.5 KM Cre was identified as *Bacillus mycoides* with a probability of 80%, while 6.4 KM Cre was *Bacillus pantothenticus* with a probability of 70%. Meanwhile, the bacterial isolate with code 6.4 M Cre was a type of Actinomyces odontolyticus with a probability of 90% and 7.4 M Cre was Actinomyces viscosus with a probability of 90%. The results of the species names of the four bacterial isolates found in the roots of *M. crenata* can be seen in Table 5.

Table 5. Species of bacterial isolate

Code of Bacterial Isolation	Species name Probability	Percentage of
5.5 KM Cre	*Bacillus mycoides*	80%
6.4 KM Cre	*Bacillus pantothenticus*	70%
6.4 M Cre	*Actinomyces odontolyticus*	90%
7.4 M Cre	*Actinomyces viscosus*	90%

Rhizobacteria are a group of bacteria found in root rhizosphere. The presence of rhizobacteria indicates of root exudates around the rhizosphere of roots. This root exudate is organic compounds in the form of amino acids, carboxylic acids and carbohydrates (Rajkumar *et al.*, 2010). Rhizobacteria can take advantage of the nutrients present in root exudates. Bacteria performs chemotaxis based on chemical signals from root exudates. This movement generally uses flagella or pili (Afzal *et al.*, 2014). From the results of the motility test, *B. mycoides, B. pantothenticus, A. odontolyticus,* and *A. viscosus* showed negative motility. Based on these results, it is shown that the four bacteria do not have locomotion in the form of pili or flagella, thus that the root exudate is only used as a nutrient for growth. In this study, four types of bacteria from two different genera were found. The number of bacteria found in rhizosphere is affected by several factors, such as the type and concentration of chemicals that act as an energy source, as well as environmental factors such as the type of substrate, temperature, pH, presence of oxygen or other acceptors, water and available nutrients (Afzal *et al.*, 2014; Rajkumar *et al.*, 2012).

Research on the process of degradation of organic matter in domestic wastewater using bacterium *Bacillus sp.* showed very good results. This study could reduce organic matter more optimally (BOD decreased by 96.67% and COD value decreased by 81.65%). This is an indication that *Bacillus sp.* uses the organic material available. Sopiah *et al.*, (2006) also stated that the high rate of degradation at the beginning of incubation indicated that microorganisms were able to grow using LAS surfactants as carbon and energy for their metabolic processes.

The process of anionic surfactant degradation carried out by bacteria requires several important stages, including terminal oxidation of the alkyl sulfatase chain with b-oxidation followed by succession, oxidation to form sulfenyl carboxylic acid, removal of sulfonates from benzene rings by desulfonation process, and cleavage of aromatic rings. and sulfonate group cleavage. The final product of LAS degradation is sulfonylenzoic acid, such as 2-4-sulfonylbenzene butyric acid (Tu *et al.*, 2020).

This study found that LAS level was decreased after 10 days of *Marsilea* treatment. From isolation and identification, four species of rhizosphere bacteria were obtained, found in the roots that play a role in the phytoremediation of detergent LAS, which were *Bacillus myocides*, *Actinomyces odontolyticus*, *Bacillus pantothenticus*, and *Actinomyces viscosus*. with the similarity coefficient respectively 80%, 90%, 70%, and 90%.

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