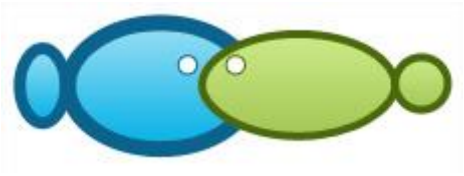

Performance of fermentation liquid from mangrove leaves (*Avicennia marina*) for controlling ice-ice disease in *Kappaphycus alvarezii* seaweed under controlled conditions

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Abstract. Fermentation liquid from mangrove leaves (*Avicennia marina*) contains secondary metabolites lactic acid, bacteriocin, and phytochemical compounds and potential endophytic bacteria. This study aimed to evaluate the capability of fermentation liquid from mangrove leaves in controlling ice-ice disease in *Kappaphycus alvarezii* seaweed under semi-field conditions. Seaweed was immersed in fermentation liquid from mangrove leaves enriched with *Bacillus subtilis* for one and two hours, respectively. Furthermore, the seaweed was tested using the pathogen that causes ice-ice disease, *Stenotrophomonas maltophilia*. The parameters evaluated in this study included seaweed bleaching, results of histopathological analysis of seaweed, growth, and morphological analysis by scanning electron microscope (SEM). The result of using fermentation liquid from mangrove leaves, enriched, or not enriched with *Bacillus subtilis*, with immersion duration between one and two hours, was no bleaching of seaweed. The use of fermented liquid was also able to improve the growth of seaweed. Fermentation liquid from mangrove leaves was able to control ice-ice disease caused by *S. maltophilia* with the best control and growth being exhibited by the treatment group with fermented liquid enriched with *B. subtilis* and two-hour immersion.
Key Words: *Bacillus subtilis*, bleaching, endophytic, histopathology, *Stenotrophomonas maltophilia*.

Introduction. Fermentation liquid from mangrove leaves (*Avicennia marina*) contains secondary metabolites, namely, lactic acid, bacteriocin, phytochemical compounds, and endophytic bacteria that are able to inhibit the bacteria that cause ice-ice disease in seaweed (Rahman et al 2020b). Ice-ice disease is believed to be caused by infection with pathogenic bacteria (Largo et al 1995), one of which is *Stenotrophomonas maltophilia*. The results of an in vitro pathogenicity test revealed that this bacterium was the main cause of ice-ice disease in *Kappaphycus alvarezii* seaweed (Achmad et al 2016).

The application of fermentation liquid from mangrove leaves has been reported to be able to inhibit the bacteria that causes ice-ice disease under in vitro conditions (Rahman et al 2020a) and the application of fermentation liquid from bael fruit (*Aegle marmelos*) has also been reported to be able to increase the growth of seaweed (Rahman & Mutalib 2015). The results of these studies showed that the fermented liquid enriched with endophytic bacteria was able to inhibit *S. maltophilia*. One of the new strategies developed to prove the success of ice-ice disease control is the application of fermented liquid enriched with potential endophytic bacteria under semi-field conditions.

The bacteria in fermented mangrove leaves liquid are endophytic bacteria derived from the mangrove leaves. Endophytic microorganisms produce secondary metabolites that can play a role in helping the host survive competition, act as a signal for interaction and communication with the host (Brader et al 2014) and be a source of nutrition for the host (Neher et al 2009). In addition to being nutritionally beneficial, endophytic bacteria

are beneficial for the morphological development and growth and the life cycle of their host (Tapia et al 2016). However, certain microorganisms can also have a detrimental effect on the host and cause diseases (Egan et al 2014).

The inhibitory activity of fermented liquid against bacteria that causes ice-ice disease was tested using disease-free seaweed micropropagules from tissue culture (Sulistiani & Yani 2014). The results obtained showed that the fermented liquid was able to inhibit the ice-ice disease of seaweed micropropagules (Rahman et al 2019a). Therefore, it is necessary to conduct further tests in semi-field and field conditions to evaluate the use of fermented *A. marina* leaves liquid for controlling ice-ice disease in *K. alvarezii* seaweed. This study is expected to propose a new strategy in the control of ice-ice disease in seaweed.

Material and Method. The study was conducted from March to September 2022. Bacterial culture and cultivation and observation of seaweed were done at the Integrated Laboratory of the Department of Aquaculture, Faculty of Fisheries, Muhammadiyah University of Luwuk. Histopathology was conducted at the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB) and observation using scanning electron microscope (SEM) was done at the Medical Laboratory, Faculty of Medicine, Hasanuddin University, Makassar.

The tested seaweed species and fermented leaves liquid. Healthy *Kappaphycus alvarezii* seaweed as the tested organism was obtained from the nursery at the seaweed cultivation center in Jaya Bakti Village, Pagimana District, Banggai Regency. The seaweed samples weighed 50-51 grams and the container used in this study was a glass aquarium measuring 46 x 32 x 28.5 cm in length, width, and height for each treatment equipped with aeration equipment (Figure 1). Before treatment, seaweed with a wet weight of 50-51 grams for each treatment was acclimatized for 3 days in an aerated container with a salinity of 32 g/L.

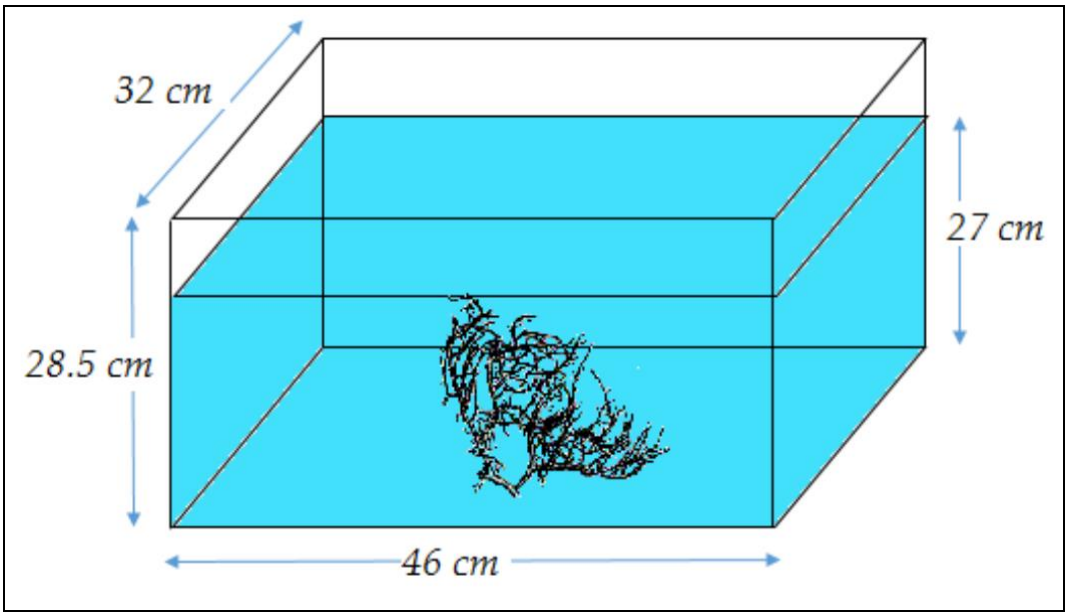

Figure 1. Depiction of an aquarium with *Kappaphycus alvarezii*.

Bacterial culture and enrichment of fermented leaves liquid. The bacteria cultured were *Stenotrophomonas maltophilia* and *Bacillus subtilis* MSAR-01. Fermented leaves liquid was enriched with *B. subtilis* MSAR-01 because it has the strongest inhibition against *S. maltophilia* (Rahman et al 2019a). *B. subtilis* MSAR-01 and *S. maltophilia* isolates were cultured on sea water complete agar (SWC agar) media (0.5 g BactoPeptone, 0.1 g yeast extract, 0.3 mL glycerol, 1.5 g Bacto™ agar, 75 mL seawater, and 25 mL of distilled water) and incubated at 28°C for 24 hours. A total of 1 inoculation loop of each bacterial isolate was taken to be cultured in liquid SWC agar, then

homogenized using a shaker at 140 rpm for 24 hours. Fermented leaves liquid was enriched with *B. Subtilis* MSAR-01 at a density of 105 cells/mL. Then, 100 µL of bacterial inoculum with a density of 105 cells/mL was inoculated into 10 mL of fermented mangrove leaves liquid.

Experimental design. This study used a completely randomized design with six treatments and three replications. The experimental design is presented in Table 1.

Table 1

Experimental design

Treatment	*Treatment*		
	Immersion Time (hour)	*Endophytic bacteria*	*Challenge test*
CB1	1	*B. subtilis* MSAR-01	*S. maltophilia*
CB2	2	*B. subtilis* MSAR-01	*S. maltophilia*
C1	1	-	*S. maltophilia*
C2	2	-	*S. maltophilia*
K^+	-	-	*S. maltophilia*
K^-	-	-	-

Test on fermented mangrove leaves liquid performance and test on seaweed resistance. Test on the resistance of seaweed to *S. maltophilia* was conducted by immersion based on each treatment (Table 1). The acclimatized seaweed was put in a research aquarium containing 15 L of seawater media with a salinity of 32 g/L and kept for one day. A total of 400 µL of pathogenic bacteria *S. maltophilia* with a density of 106 cells/mL were collected from the bacterial culture and suspended in each treatment. Thallus visual characteristics were observed daily (Largo et al 1995; Achmad et al 2016; Rahman et al 2019b).

Test Parameters

Method of measurement of growth and observation of morphological changes of seaweed. Changes in weight were measured by weighing the seaweed from the beginning of cultivation to the end of cultivation. Observations of morphological changes and measurements of growth in seaweed were conducted every week after infection. Later, the morphological changes were observed by taking photos of seaweed at a distance of 15 cm under the same lighting. Furthermore, the photos were analyzed descriptively.

Histopathology and scanning electron microscopy (SEM). The preparation process for seaweed histopathology was done by seaweed tissue fixation using buffered neutral formalin (BNF) and dehydration using graded series of alcohol solution, namely 70, 80, 90, 95 and 100%. The seaweed parts were then cleared using xylene and impregnated with liquid paraffin to make a solid block. Paraffin block containing tissue was cut with a 6 µm microtome. Specimen staining was done with hematoxylin and eosin. Furthermore, observations were made with a microscope at a magnification of 40X (Achmad et al 2016; Rahman et al 2019a). The preparation procedure for SEM observation of seaweed tissue was in accordance with the procedure proposed by Goldstein et al (1992).

Data analysis. Data on morphological change, which was bleaching of seaweed tissue, seaweed histopathology, and SEM micrographs of seaweed tissue were analyzed descriptively, while the level of resistance of seaweed to *S. maltophilia* was analyzed using one-way ANOVA. If there is a significant effect ($p<0.05$), then the analysis is continued with Tukey test using SPSS version 24 software.

Results and Discussion

Experiment 1*, *performance of seaweed against S. maltophilia. The results of the analysis of variance showed that fermented mangrove leaves liquid had an effect on the growth and resistance of seaweed to *S. maltophilia* infection. The growth of seaweed immersed in fermented mangrove leaves liquid was significantly different from the growth of seaweed that was not immersed in one but infected with *S. maltophilia* (Table 2).

Table 2

Average daily growth rate of seaweed in each treatment during the study

Treatment	*Average daily specific growth (%) ($\overline{X}$ ± SD)*
K^+	-3.14 ± 0.00[a]
K^-	1.42 ± 0.20[b]
CB1	2.27 ± 0.05[c]
CB2	2.34 ± 0.02[c]
C1	1.77 ± 0.08[d]
C2	1.95 ± 0.17[d]

Note: The initial weight of the seaweed samples was 50-51 grams. Different letters in the mean value (±standard deviation) in the same column indicate a significant difference at the 5% significance level.

The result of the Tukey's test showed that the positive control (K^+) had a significant effect on the negative control K^-, CB1, CB2, C1, and C2. Treatment CB1 did not have a significant effect on treatment CB2 but had a significant effect on treatments C1 and C2. The treatment C1 did not have a significant effect on C2. Based on Table 2 above, the daily weight growth of seaweed *Kappaphycus alvarezii* in the treatment group CB2 (2.34 ± 0.02%) had the highest percentage value, followed by the daily weight growth percentage of seaweed in the treatment group CB1 (2.27 ± 0.05%), treatment group C2 (1.95 ± 0.17%), and the treatment group C1 (1.77 ± 0.08%).

Bacterial infection without immersion in fermented mangrove leaves liquid had a significant effect on the weight loss of seaweed. The content of nutrients and compounds in fermented mangrove leave liquid plays an important role in the growth of seaweed and the control of *S. maltophilia*, which causes seaweed ice-ice disease. The content of macro and micronutrients in fermented mangrove leave liquid can increase the growth of seaweed. There was an increase in the weight of seaweed in the treatment of seaweed immersed in fermented mangrove leaves liquid, which is thought to be due to the role of primary metabolite compounds present in fermented mangrove leaves liquid in the form of macro and micronutrients, that can increase the growth of seaweed (Rahman et al 2019b; Rahman et al 2021). In a study by Rahman and Mutalib (2015), fermented liquid from bael fruit (*Aegle marmelos*) using local microorganisms was able to increase the growth of seaweed. One of the causes of the decreased growth of seaweed is pathogenic bacteria. Darma et al (2021) stated that pathogenic infection in seaweed thallus is one of the causes of a decrease in seaweed weight and lower levels of carrageenan in seaweed thallus. Furthermore, Vairappan (2006) said that pathogenic bacteria that infect seaweed thallus degrade carrageenan that can be extracted from seaweed.

Observation on changes in the morphology of seaweed in all treatments of immersion with fermented liquid, both those without endophytic bacteria and endophytic bacteria, did not discover any symptoms of bleaching. Seaweed bleaching occurred in seaweed in the positive control treatment group (K^+) (Figure 2) that was infected with *S. maltophilia* and was not immersed in fermented mangrove leaves liquid. It was observed for only 4 days because the seaweed underwent 100 percent bleaching and even started to fall off and crumble. Meanwhile, seaweed in other treatment groups did not experience symptoms of ice-ice disease, so the observations were continued for 40 days.

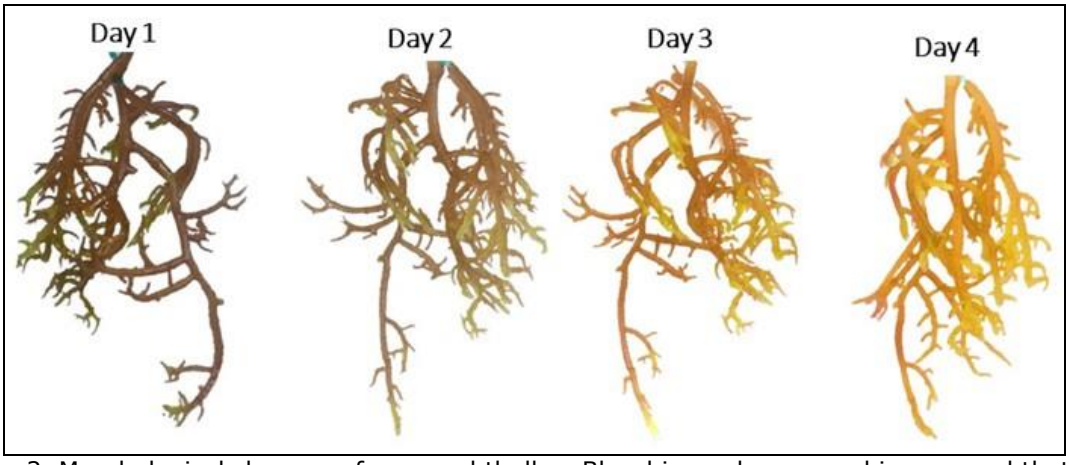

Figure 2. Morphological changes of seaweed thallus. Bleaching only occurred in seaweed that was not immersed in fermented mangrove leaves liquid (K^+). Seaweed thallus in other treatment groups did not show any morphological changes.

Immersion of seaweed in fermented mangrove leaves liquid and enrichment with endophytic bacteria were able to control ice-ice disease, indicated by the test for *S. maltophilia* infection that did not show any symptoms of bleaching. This is in line with the results of test on seaweed micropropagules against bacteria that cause ice-ice disease conducted by Rahman et al (2019b), which discovered that fermented mangrove leaves liquid was able to inhibit ice-ice disease in seaweed micropropagules. Fermented liquid contains secondary metabolites of lactic acid, bacteriocin, and phytochemical compounds and endophytic bacteria that are able to inhibit the bacteria that causes ice-ice disease in seaweed (Rahman et al 2019a). Different results were exhibited by the positive control treatment group (K^+) that was not immersed in the fermented liquid where the seaweed had ice-ice disease. According to Steinberg et al (2002), bleaching occurs because of a decrease in the content of furanone metabolites. Furanone is a chemical defense mechanism of seaweed, that decrease tissue necrosis occurrence and death (Fernandes et al 2011).

Experiment 2, histopathological and scanning electron microscopy (SEM) observations of seaweed thallus. The histopathological conditions of seaweed thallus that was immersed in fermented liquid with enrichment of *B. subtilis* and tested for *S. maltophilia* (CB), seaweed thallus without immersion in fermented liquid and without test for *S. maltophilia* (K^-) test, and seaweed thallus that was not immersed in the fermented liquid and not tested for *S. maltophilia* (K+) are presented in Figure 3. Histopathologically, thalli in the treatment groups CB and K^- had normal tissue, while the control group K^+ indicated very severe tissue damage. The protoplasm (Ep) in the bleached thallus tissue began to disappear and there were intercellular spaces (CW).

Observation using SEM showed the presence of a bacterial population in the seaweed tissue. The results of observations using SEM on the best treatment group (CB) and the control groups K^- and K^+ (Figure 4) showed that the seaweed tissue immersed in fermented mangrove leaves liquid enriched with *B. subtilis* endophytic bacteria contained a population of *B. subtilis* bacteria, while seaweed that was not immersed in fermented liquid, but tested for *S. maltophilia*, was presumably dominated by *S. maltophilia*.

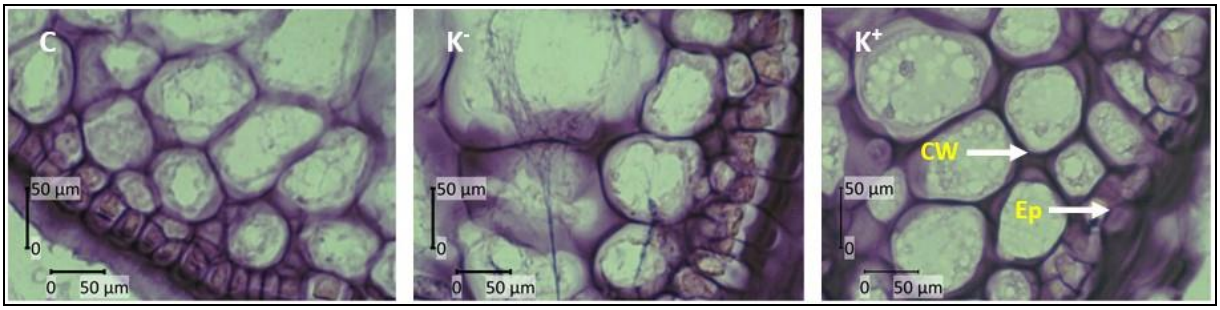

Figure 3. Histology of seaweed thalli. CB, the thallus tissue was normal (the distance between cells was small); K^-, the condition of the thallus tissue was not tested (distance between cells was small); K^+, thallus tissue was damaged (distance between cells was long) (H&E staining, 40x magnification).

Tissue damage in the control group K^+ was caused by pathogens. The process of pathogenic infection starts from the epidermis and then the pathogen enters other tissues. The thallus tissue in the CB and K^- treatment groups was healthy. Healthy tissue has perfect carrageenan and cellulose, which are able to protect the cell walls of seaweed. Disease infection can cause changes in tissue structure (Magi et al 2009; Senapin et al 2018). This is in line with Lundsor (2001) who stated that healthy thalli are able to protect the cell walls of seaweed, while thallus infected with ice-ice disease contains little or no carrageenan and cellulose. According to Aris and Labenua (2020), healthy seaweed tissue has components of constituent cells of tissue, such as cortical cells and modular cells, which are still intact, compact, and well positioned, while the structure of seaweed tissue infected with ice-ice disease is damaged or subjected to lysis, resulting in pigment loss (Hayashi et al 2008; Maulani et al 2018).

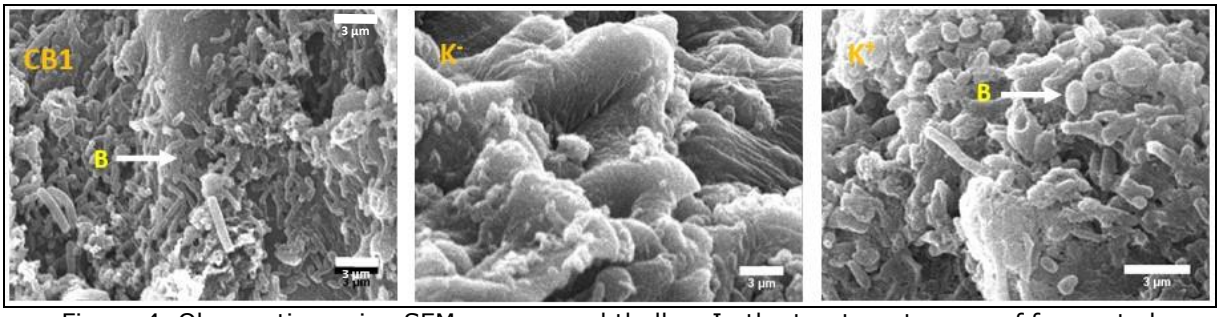

Figure 4. Observation using SEM on seaweed thallus. In the treatment group of fermented mangrove leaves liquid enriched with *B. subtilis* MSAR-01 with immersion duration of one hour (CB1), the colonies of *B. subtilis* were very dense; in the control group without infection (K^-), no bacteria and *S. maltophilia* infection were found; in the control group without immersion and with *S. maltophilia* infection (K^+), *S. maltophilia* colonies were very dense (B = bacteria).

Seaweed thalli in the treatment group CB1 and control groups K^+ and K^-, which were observed using SEM, exhibited differences in bacterial colonization. Seaweed thallus in the treatment group enriched with *B. subtilis* (CB1) showed the presence of *Bacillus* bacteria colonization, while in the control group K^+, the colony of *S. maltophilia* and other types of pathogenic bacteria was very dense. Seaweed thallus in the control group K^- did not show any colony of bacteria.

The bacteria discovered in the control group K^+ were not only *S. maltophilia* but also other pathogenic bacteria because generally, the bacteria that damage tissue are pathogenic bacteria. The same thing was also found in the treatment group CB1, which besides being colonized by *B. subtilis*, was also thought to be colonized by other beneficial endophytic bacteria. Pathogenic bacteria infect seaweed by degrading its chemical component called carrageenan (Vairappan 2006).

Conclusions. The fermentation liquid from mangrove leaves of *Avicennia marina* was able to control ice-ice disease caused by *Stenotrophomonas maltophilia* with the best

control and growth being exhibited by the treatment of fermented liquid enriched with *Bacillus subtilis* with an immersion duration of 2 hours.

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Conflict of interest. The authors declare that there is no conflict of interest.

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