KORESPONDENSI PAPER

JUDUL. : Antibacterial Activity of Microalgae *Dunaliella salina*, *Tetraselmis chuii* and *Isochrysis affinis galbana* Tahitian clone Against Aquatic Pathogens

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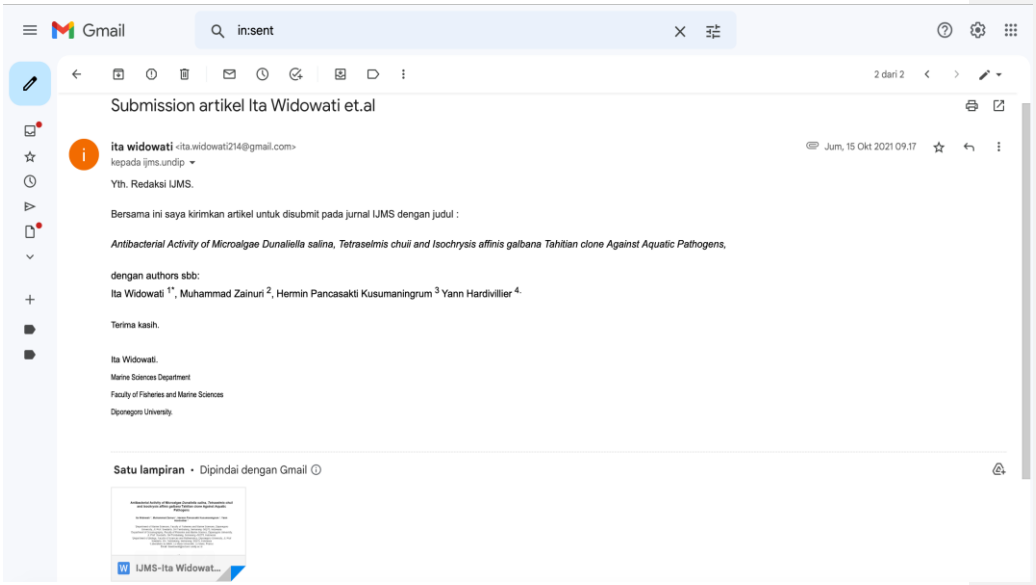
1. **Submission Artikel (15 Oktober 2021)**



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INITIAL MANUSCRIPT
(15 Oktober 2021)

Antibacterial Activity of Microalgae *Dunaliella salina*, *Tetraselmis chuii* and *Isochrysis affinis galbana* Tahitian clone Against Aquatic Pathogens

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Abstract

The crude methanol extracts of three microalgae, *Dunaliella salina*, *Tetraselmis chuii*, *and Isochrysis* affinis *galbana* Tahitian clone, have been investigated for antibacterial activity using the disk diffusion method against aquatic pathogens of fish, shrimp, and shellfish *e.g. Pseudomonas fluorescence* and *Vibrio harveyi*. This research aimed to know the antibacterial activity of crude extract of these microalgae against aquatic pathogens. The disk diffusion method was used to investigate the antibacterial activity. The result showed that *T. chuii* has the inhibition zone for both tested bacteria *P. fluorescence* and *V. harveyi* while *D. salina* and *I.* affinis *galbana* Tahitian clone have inhibition zone only for *V. harveyi*. These three microalgae may have potential use in aquatic pathogens as antimicrobial agents. It would be possible to develop biologically active compounds of microalgae as a functional feed for aquaculture.

Keywords*: Dunaliella salina*, *Tetraselmis chuii*, *Isochrysis* affinis *galbana* Tahitian clone, antibacterial activity, aquatic pathogens.

Introduction

The problems of microbial resistance in aquaculture have led to a search for new antimicrobial compounds. The major issues of current antimicrobial agents are toxicity, lack of efficacy, inhibiting cost, and their frequent use leading to the emergence of resistant strains. Thus, there is an urgent need to search for alternative biodegradable agents, which should be free from

side effects. It is generally that natural compounds are biodegradable and environmentally acceptable. Microalgae are mainly utilized in aquaculture particularly due to their nutrition contents (Khatoon *et al*, 2014; Hoai Thu *et al*, 2015). Besides nutrition advantages, microalgae also have been explored for the use of pigments and other biological purposes. Various species of microalgae are known as an essential food source in the rearing of all stages of marine bivalve mollusks (clams, oysters, and scallops), and of the post-larval stages of some marine gastropods (*e.g.* abalone), as well as larvae of several marine fish species, penaeid shrimp and zooplankton. There are three different groups of live diets, commonly used in commercial larvae culture of fish and shellfish: 1). Different species of microalgae ranging between 2 µm and 20 µm in size for bivalves, penaeid shrimps, rotifers, copepods, and fish; 2). The rotifers *Brachionus plicatilis* and *B. rotundiformis* (50 µm to 200 µm) in size for crustaceans and marine fish; 3) The brine shrimp *Artemia* sp. nauplii (400µm to 800 µm) in size for crustaceans and fish (Lavens & Sorgeloos, 1996).

Although the bioactive compound also can be found in macroalgae such as *Sargassum* sp. (antibacterial, antiviral, antioxidants) (Hardouin *et al.*, 2013; Widowati *et al.*, 2014; Susilowati *et al.*, 2015). However, the application of macroalgae for aquaculture is being limited, for example, it cannot be used as feed for larval stages. Microalgae have rich sources of structurally and biologically active metabolites including antioxidants (Widowati *et al.*, 2017) and antibiotics which inhibit bacteria responsible for fish, shellfish, and human pathogens (Pradhan *et al.*, 2011; Najdenski *et al.*, 2013). Secondary or primary metabolites produced by these microorganisms may be potential bioactive compounds of interest in aquafeed as an antimicrobial agent (Pradhan *et al.*, 2011). Therefore, some microalgae have been suggested as a new functional feed ingredient (Becker, 2004). This research aimed to determine the antibacterial activity of three species microalgae *Dunaliella salina* (Teodoresco, 1905), *Tetraselmis chuii* (Butcher, 1959), and *Isochrysis* affinis *galbana* Tahitian clone (T-Iso) against aquatic pathogens *Pseudomonas fluorescence* (Flügge, 1886) and *Vibrio harveyi* (Johnson & Shunk, 1936; Baumann *et al.*, 1981).

Materials and Methods

Culture of Microalgae

Three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis* affinis *galbana* Tahitian clone (T-Iso) was cultured by using batch continuous culture with the comparison between algae and seawater was 1 L : 3 L. Walne was used for the nutritional compound for the culture compared by volume of culture 1 mL: 1 L with luminous intensity 1500 lux to 3000 lux and 23°C to 25 °C for temperature (Andersen, 2005).

The density of the culture microalgae was counted with a hemocytometer by using a binocular microscope. The density of the microalgae was counted every day. The microalgae were homogenized first before putting the pipet into the hemocytometer and close with the cover glass. Then, it was observed under a microscope and start to count with the equation below:

$$N = \frac{(N1+N2)}{2} x \frac{1}{10.2\,mm^2\, x\, 0.1mm} x \frac{1mm^3}{10^{-3}\,mL} \quad (1)$$

Note:

N: Cell density (cell. mL^{-1})

N1: Total cell in 80 small squares (replica 1)

N2: Total cell in 80 small squares (replica 2)

0.2 mm: Wide of hemocytometer in 80 squares

0.1 mm: The depth liquid on a hemocytometer.

The microalgae were harvested on the stationary phase of culture. The biomass of microalgae was obtained with a centrifuge at 5000 rpm (1 rpm equal 1/60 Hz) for 10 min and then dry in a room at view days, then called dry biomass (Mishra *et al.*, 2012).

Extraction

Extraction involves the separation of plant or animal tissues from inactive or inert components by using selective solvents in standard extraction procedures. Dry biomass was extracted by using methanol solvent with sonification at 50 Hz for 15 min. Then, the solvents were evaporated by using

rotary evaporation until there is no solvent called with crude extract bioactive of microalgae (Trianto *et al.*, 2011).

Antibacterial Activities

The screening of antibacterial activities from extract microalgae against two aquatic pathogens bacteria *Vibrio harveyi* and *Pseudomonas fluorescence* was performed by using the disk diffusion method.

The extract of each microalga was diluted into three concentrations *Dunaliella salina* 13.32 µg. g^{-1},1.33 µg. g^{-1}, and 0.13 µg. g^{-1}, *Tetraselmis chuii* 12.61 µg. g^{-1},1.26 µg. g^{-1}, and 0.13 µg. g^{-1}, T-Iso 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}. One of the 50 µL culture of tested bacteria in logarithmic phase were spread on to agar medium. Several paper disks (8 mm; Advantec Toyo Roshi, Ltd, Japan) containing 30 µL of each concentration were placed on the respective agar surface. The plates were incubated at room temperature for 48 h. Antibacterial activity was defined by inhibition zones around the paper disk (Radjasa *et al.*, 2009).

Results and Discussion

Culture of Microalgae

Microalgae were counted for the density every day with a hemocytometer under the microscope binocular. The result of the density of microalgae *Dunaliella salina*, *Tetraselmis chuii,* and T-Iso is shown in Figure 1.

Extraction

The microalgae were harvested on the stationary phase of culture. The results of biomass of microalgae were presented in Table 1, while the result of extract biomass with methanol solvent is shown in Table 2.

The potential of extract microalgae to produce substances inhibiting the growth of selected bacteria was evaluated in this research to support the efforts for fulfilling the important need for

aquaculture purposes. Antibacterial activities of three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and T-Iso against two species of bacterias aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence* was carried out using the disk diffusion method. The range of the concentrations, *Dunaliella salina* were 13.32 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}, *Tetraselmis chuii* were 12.61 µg. g^{-1}, 1.26 µg. g^{-1}, and 0.13 µg. g^{-1}, and T-Iso were 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}, was conducted for the crude extract from methanol solvent of microalgae. Based on the result, the best concentration will be used for the next purification extracted with another solvent.

Furthermore, the organic polar solvent provided extraction yield, ranging from 0.33 g for *Dunaliella salina*, 0.43 g for *Tetraselmis chuii*, and 1.69 g for T-Iso. It was obtained from 3 L and 1.5 L of culture. The crude extract for methanol solvent with high polarity was able to extract the antimicrobial compound. Several authors (Borowitzka, 1995, Ozdemir *et al*., 2004) have attributed the cyanobacteria antimicrobial activity to different compounds.

Antibacterial Activities

The antibacterial activities were carried out by using disk diffusion methods. The tested bacteria were counted for their density by using the McFarland standards method (Table 3). The result of antibacterial activity by using the disk diffusion method against *Vibrio harveyi* is shown in Table 4 and against *Pseudomonas fluorescence* is shown in Table 5.

Antibacterial activity of various extracts of the microalgae *Spirulina platensis* (Pradhan *et al*., 2011), *Euglena viridis* (Das *et al.*, 2005), cyanobacteria (Najdenski *et al*., 2013), have been reported as the main groups of microalgae to produce antimicrobial substances.

The result of antibacterial activities in this study showed that only *Tetraselmis chuii* provided the inhibition zone against two tested bacteria *V. harveyi* with the inhibition zone 3.0 mm ± 0.6 mm on 100 µg. g^{-1} of extract concentration and 4.2 mm ± 1.1 mm against *P. fluorescence* on 10 000 µg. g^{-1} of extract concentration. The result is similar to the finding that methanol extracts of *Tetraselmis* sp. showed maximum zone of inhibition against *Pseudomonas* sp (Rajendran, *et al*, 2014). Furthermore, Kokou *et al* (2012) found that *Tetraselmis chui* and *Isochrysis* sp showed antibacterial

activity against six (6) Vibrio bacterial strains: *V. parahaemolyticus, V. anguillarum, V. splendidus, V. scophthalmi, V. alginolyticus,* and *V. lentus*. Besides antibacterial activity, *Tetraselmis chui* is an important producer of fatty acid, and this is an insignificant relationship with environmental conditions (Mohammadi *et.al*, 2015).

Meanwhile, two microalgae, *Dunaliella salina* and T-Iso tested*,* have the inhibition zone only against *V. harveyi* with the inhibition zone 4.4 mm ± 0.6 mm and 3.2 mm ± 0.7 mm on 10 000 µg. g^{-1} of extract concentration.

This result is in agreement with the finding of Molina-Cárdenas *et.al* (2014) that demonstrate *Isochrysis galbana* synthesizes antibacterial fatty acids that inhibit the growth of pathogenic bacteria such as *V. harveyi*, *V. alginolyticus,* and *V. campbellii*.

The antimicrobial compounds of microalgae are expected due to their lipids contains. Lipids and some free fatty acids are known to show antibacterial activities from algae (Desbois & Smith, 2010; Plaza *et al.*, 2010). It has been shown that the promoting effect on membrane damage leads to a leakage of molecules from the microbial cells, reduction in nutrient uptake, or inhibition of cellular respiration (Smith *et al.*, 2010). An antibiotically active fatty acid is presented in a high concentration in algae as stated by Hoai Thu *et.al* (2015), found that *I. galbana* Parke, strain HP has the highest content of Docosahexaenoic acid (DHA), up to 14.7% of total fatty acid and maximal Polyunsaturated fatty acids (PUFAs) values at the early stationary phase. The primary PUFAs are stearidonic acid (18:4n-3) (Nalder *et.al*., 2015).

Conclusion

The crude extract of three species microalgae, *Dunaliella salina*, *Tetraselmis chuii,* and *Isochrysis* affinis *galbana* Tahitian clone*,* can be used as antibacterial activity against aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence*. The best result for the aquatic purpose was *T. chuii* which can inhibit both pathogenic bacteria *V. harveyi* and *P. fluorescence* and also have antibacterial activities for three replicas. Hence, it is necessary to carry out further research about the availability of microalgae resources as a new functional feed for aquaculture.

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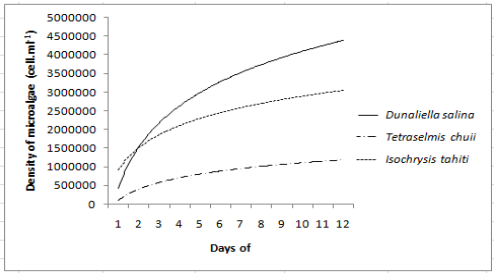


Figure 1: Density of *Dunaliella salina*, *Tetraselmis chuii*, and *T*-Iso (cell. mL^{-1}) x 10^4.

Table 1. The biomass of microalgae

No. Species	Wet biomass (g)	Dry biomass (g)
1. *D. salina*	6.65	1.026
2. *T. chuii*	7.97	1.23
3. T-Iso	71.74	3.54

Table 2. The result of crude extract of microalgae with methanol solvent

No.	Species	Dry biomass *(g)*	Extract biomass *(g)*
1.	*D. salina*	1.026	0.33
2.	*T. chuii*	1.230	0.43
3.	T-Iso	3.540	1.69

Table 3. Results of McFarland standard for bacteria

No.	Bacteria	OD	Cell density (cell · mL^{-1})
1.	*Vibrio harveyi*	0.671	12 x 10^8
2.	*Pseudomonas fluorescence*	0.107	1.5 x 10^8

Note : OD: Optical Density

Table 4. Antibacterial activity of microalgae against *Vibrio harveyi*

No	Species	Inhibition zone (mm)					
		24 h			48 h		
		a*)	b*)	c*)	a*)	b*)	c*)
1	*Dunaliella salina*	4.4±0.6	1.8±0.4	0	4.3±0.9	1.8±0.3	0
2	*Tetraselmis chuii*	3.5±0.7	2.3±0.3	3.0±0.6	3.5±0.7	2.2±0.3	2.9±0.5

3	*T*. Iso	3.2±0.7	2.9±0.2	2.5±0.8	3.0±0.6	2.9±0.2	2.5±0.7

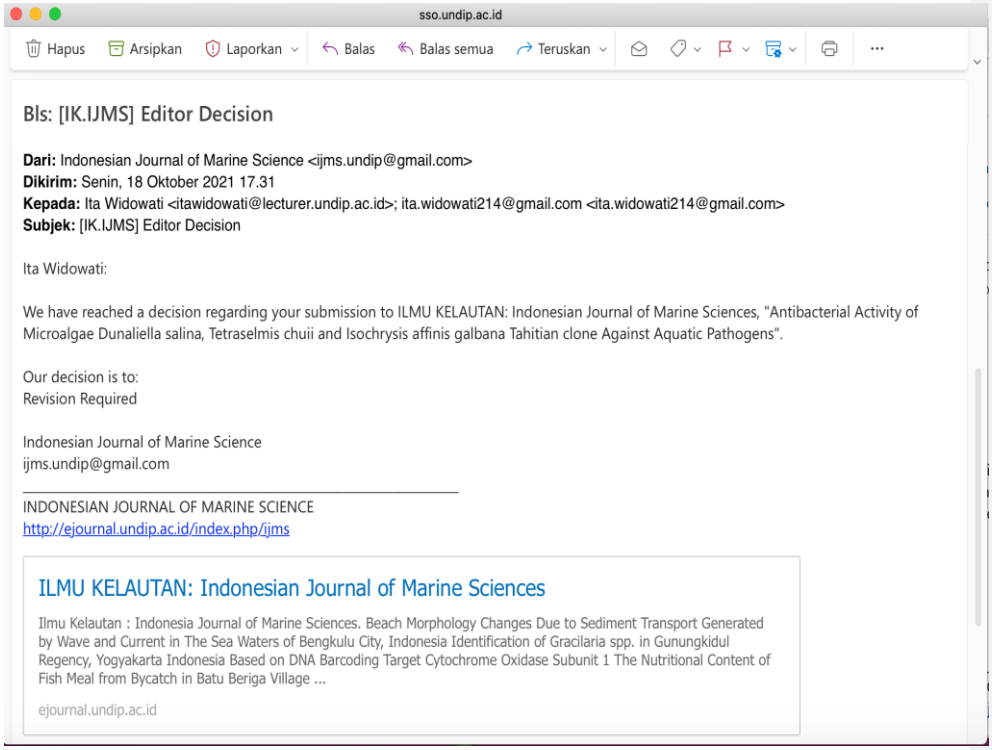
Note:
Concentration of *V harveyi* for
a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}
*) Mean ± SD

Table 5. Antibacterial activity of microalgae against *Pseudomonas fluorescence.*

No	Species	Inhibition zone (mm)					
		24 h			48 h		
		a*)	b*)	c*)	a*)	b*)	c*)
1	*Dunaliella salina*	0.0	0.0	0.0	0.0	0.0	0.0
2	*Tetraselmis chuii*[*)]	4.2 ± 1.1	3.2 ± 0.5	3.1 ± 0.1	4.2 ± 0.9	2.9 ± 0.2	3.1 ± 0.2
3	*T*-Iso	0.0	0.0	0.0	0.0	0.0	0.0

Note:
Concentration of *P. fluorescence* for
a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}
*) Mean ± SD

3. Editor Decision: Revision Required (18 Oktober 2021)



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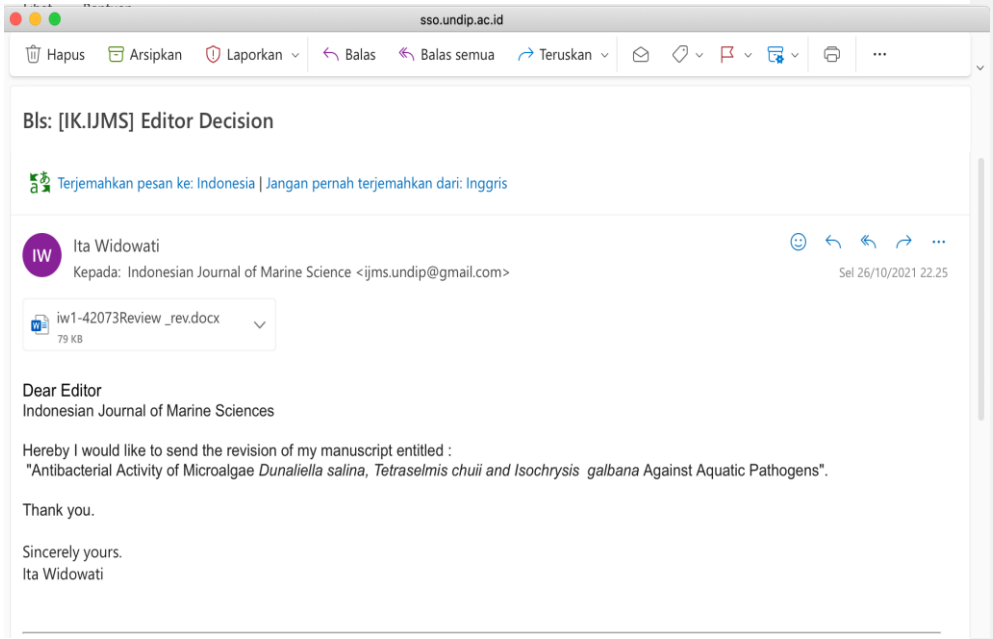

FILE REVISI #1
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Antibacterial Activity of Microalgae *Dunaliella salina*, *Tetraselmis chuii* and *Isochrysis galbana* Against Aquatic Pathogens

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Abstract

Recently, expanded consideration has been paid to the business and potentiality of microalgae. Some microalgae are at present being studied for their capacity to find important metabolites for the drug industry or aquacultural applications. Concerning these biotechnological challenges, there is a consistent exertion accommodated in both finding and taking advantage of new microalgal assets and fostering their putative business results or modern valorizations. The crude methanol extracts of three microalgae, *Dunaliella salina*, *Tetraselmis chuii*, *and Isochrysis galbana,* have been investigated for antibacterial activity using the disk diffusion method against aquatic pathogens of fish, shrimp, and shellfish *e.g. Pseudomonas fluorescence* and *Vibrio harveyi*. This research aimed to analyze the antibacterial activity of crude extract of these microalgae against aquatic pathogens. The disk diffusion method was used to investigate the antibacterial activity. The result showed that only *T. chuii* has the inhibition zone for both tested bacteria *P. fluorescence* and *V. harveyi* with the inhibition zone of 3.0 ± 0.6 mm on 100 µg. g^{-1} of extract concentration and 4.20 ± 1.1 mm against *P. fluorescence* on 10000 µg. g^{-1} of extract concentration. While *D. salina* and *I. galbana* have inhibition zone only for *V. harveyi* with the inhibition zone of 4.4 ± 0.6 mm and 3.2 ± 0.7 mm on 10000 µg. g^{-1} of extract concentration. These three microalgae may have potential use in aquatic pathogens as antimicrobial agents. It would be possible to develop biologically active compounds of microalgae as a functional feed for aquaculture.

***Keywords**:* antibacterial activity, aquatic pathogens, marine microalgae, microalgal extract.

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Introduction

The problems of microbial resistance in aquaculture have led to a search for new antimicrobial compounds. The significant issues of current antimicrobial agents are harmfulness,

absence of adequacy, restraining cost, and their frequent use leading to the emergence of resistant strains. Subsequently, there is an earnest need to look for elective biodegradable agents, which should be free from side effects. It is generally that natural compounds are biodegradable and eco-friendly. Microalgae are mainly utilized in aquaculture particularly due to their nutrition contents (Khatoon *et al*, 2014; Hoai Thu *et al*, 2015). Besides nutrition advantages, microalgae also have been explored for the use of pigments and other biological purposes. Different species of microalgae are known as a fundamental food source in the rearing of all stages of marine bivalve mollusks (clams, oysters, and scallops), and of the post-larval stages of some marine gastropods (*e.g.* abalone), larvae of marine fish, penaeid shrimp, and zooplankton. There are three distinct types of live feed, usually utilized in commercial larvae culture of fish and shellfish: 1). Different species of microalgae ranging between 2 µm and 20 µm in size for bivalves, penaeid shrimps, rotifers, copepods, and fish; 2). The rotifers *Brachionus plicatilis* and *B. rotundiformis* (50 to 200 µm) in size for crustaceans and marine fish; 3) The brine shrimp *Artemia* sp. nauplii (400 to 800 µm) in size for crustaceans and fish (Lavens & Sorgeloos, 1996).

The bioactive compound also can be found in macroalgae such as *Sargassum* sp. (antibacterial, antiviral, antioxidants) (Hardouin *et al.*, 2013; Widowati *et al.*, 2014; Susilowati *et al.*, 2015). However, the application of macroalgae for aquaculture is being limited, for example, it cannot be used as feed for larval stages. Microalgae have rich sources of structurally and biologically active metabolites including antioxidants (Widowati *et al.*, 2017) and antibiotics which inhibit bacteria responsible for fish, shellfish, and human pathogens (Pradhan *et al.*, 2011; Najdenski *et al.*, 2013). Secondary or primary metabolites produced by these microorganisms may be potential bioactive compounds of interest in aquafeed as an antimicrobial agent (Pradhan *et al.*, 2011). Therefore, some microalgae have been suggested as a new functional feed ingredient (Becker, 2004). This research aimed to determine the antibacterial activity of three species microalgae *Dunaliella salina* (Teodoresco, 1905), *Tetraselmis chuii* (Butcher, 1959), and *Isochrysis galbana* against aquatic pathogens *Pseudomonas fluorescence* (Flügge, 1886) and *Vibrio harveyi* (Johnson & Shunk, 1936; Baumann *et al.*, 1981).

Materials and Methods

Culture of Microalgae

Three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* was cultured by using batch continuous culture with the comparison between algae and seawater was 1 : 3 L. Walne was used for the nutritional compound for the culture compared by volume of culture 1 mL: 1 L with luminous intensity 1500 lux to 3000 lux and 23 to 25 °C for temperature (Harrison & Berges, 2005).

The density of the culture microalgae was counted with a Neubauer hemocytometer by using a binocular microscope. The density of the microalgae was counted every day. The microalgae were homogenized first before putting the pipet into the hemocytometer and closed with the cover glass. Then, it was observed under a microscope and started to count with the equation (Hadioetomo, 1993):

$$N = \frac{(N1+N2)}{2} x \frac{1}{10.2\,mm^2\,x\,0.1mm} x \frac{1mm^3}{10^{-3}\,mL} \quad (1)$$

Note:

N: Cell density (cell. mL^{-1})

N1: Total cell in 80 small squares (replica 1)

N2: Total cell in 80 small squares (replica 2)

0.2 mm: Wide of hemocytometer in 80 squares

0.1 mm: The depth liquid on a hemocytometer.

The microalgae were harvested on the stationary phase of culture. The biomass of microalgae was obtained with a centrifuge at 5000 rpm (1 rpm equal 1/60 Hz) for 10 min and then dried in a room at view days, then called dry biomass (Mishra *et al.*, 2012).

Extraction

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Extraction involves the separation of plant or animal tissues from inactive or inert components by using selective solvents in standard extraction procedures. Dry biomass was extracted by using methanol solvent with sonification at 50 Hz for 15 min. Then, the solvents were evaporated by using rotary evaporation until there is no solvent called with crude extract bioactive of microalgae (Trianto *et al.*, 2011).

Antibacterial Activities

The screening of antibacterial activities from extract microalgae against two aquatic pathogens bacteria *Vibrio harveyi* and *Pseudomonas fluorescence* was performed by using the disk diffusion method.

The extract of each microalga was diluted into three concentrations *Dunaliella salina* 13.32 µg. g^{-1},1.33 µg. g^{-1}, and 0.13 µg. g^{-1}; *Tetraselmis chuii* 12.61 µg. g^{-1},1.26 µg. g^{-1}, and 0.13 µg. g^{-1}; *Isochrysis galbana* 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}. One of the 50 µL culture of tested bacteria in the logarithmic phase were spread on to agar medium. Several paper disks (8 mm; Advantec Toyo Roshi, Ltd, Japan) containing 30 µL of each concentration were placed on the respective agar surface. The plates were incubated at room temperature for 48 h. Antibacterial activity was defined by inhibition zones around the paper disk (Radjasa *et al.*, 2009).

Results and Discussion

Culture of Microalgae

Microalgae were counted for the density every day with a hemocytometer under the microscope binocular. The highest densities are found in *Dunaliella salina* (4.4 x 10^6 cells.mL^{-1}), followed by *Isochrysis galbana* (3.2 x10^6 cells.mL^{-1}), and the lowest is in *Tetraselmis chuii* (0.96 x 10^6 cells.mL^{-1}). However, the highest dry biomass is found in *I. galbana* (3.54 g) and the lowest is in *D. salina* (1.026 g). The highest density in *D. salina* is not in line with the dry biomass, this may be due to the cell size since *D. salina* cell is (2.8-40 µm, Borovkov et.al, 2019) and *I. galbana* cell size is 4-6 µm (Cordoba-Matson, 2013). The cell size could change with growth, light intensity, and different condition (Borowitzka, 2021). The result of the density of microalgae *Dunaliella salina*, *Tetraselmis chuii,* and *I. galbana* is shown in Figure 1.

Extraction

The microalgae were harvested on the stationary phase of culture. The results of biomass of microalgae were presented in Table 1, while the result of extract biomass with methanol solvent is shown in Table 2.

The potential of extract microalgae to produce substances inhibiting the growth of selected bacteria was evaluated in this research to support the efforts for fulfilling the important need for aquaculture purposes. Antibacterial activities of three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* against two species of bacteria's aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence* was carried out using the disk diffusion method. The range of the concentrations, *Dunaliella salina* were 13.32 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}; *Tetraselmis chuii* were 12.61 µg. g^{-1}, 1.26 µg. g^{-1}, and 0.13 µg. g^{-1}; and *Isochrysis galbana* were 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}, respectively was conducted for the crude extract from methanol solvent of microalgae. Based on the result, the best concentration will be used for the next purification extracted with another solvent.

Furthermore, the organic polar solvent provided extraction yield, ranging from 0.33 g for *Dunaliella salina*, 0.43 g for *Tetraselmis chuii*, and 1.69 g for *Isochrysis galbana.* It was obtained from 3 L and 1.5 L of culture. The crude extract for methanol solvent with high polarity was able to extract the antimicrobial compound. Several authors (Borowitzka, 1995, Ozdemir *et al*., 2004) have attributed the cyanobacteria antimicrobial activity to different compounds.

Antibacterial Activities

The antibacterial activities were carried out by using disk diffusion methods. The tested bacteria were counted for their density by using the McFarland standards method (Table 3). The result of antibacterial activity by using the disk diffusion method against *Vibrio harveyi* is shown in Table 4 and against *Pseudomonas fluorescence* is shown in Table 5.

Antibacterial activity of various extracts of the microalgae *Spirulina platensis* (Pradhan *et al*., 2011), *Euglena viridis* (Das *et al.*, 2005), cyanobacteria (Najdenski *et al*., 2013), have been reported as the main groups of microalgae to produce antimicrobial substances.

The result of antibacterial activities in this study showed that only *Tetraselmis chuii* provided the inhibition zone against two tested bacteria *V. harveyi* with the inhibition zone 3.0 mm ± 0.6 mm on 100 µg. g^{-1} of extract concentration and 4.2 mm ± 1.1 mm against *P. fluorescence* on 10 000 µg. g^{-1} of extract concentration. The result is similar to the finding that methanol extracts of *Tetraselmis* sp. showed maximum zone of inhibition against *Pseudomonas* sp (Rajendran, *et al*, 2014). Furthermore, Kokou *et al* (2012) found that *Tetraselmis chui* and *Isochrysis* sp showed antibacterial activity against six (6) Vibrio bacterial strains: *V. parahaemolyticus, V. anguillarum, V. splendidus, V. scophthalmi, V. alginolyticus,* and *V. lentus*. Besides antibacterial activity, *Tetraselmis chui* is an important producer of fatty acid, and this is an insignificant relationship with environmental conditions (Mohammadi *et.al*, 2015).

Meanwhile, two microalgae, *Dunaliella salina* and *Isochrysis galbana* tested, have the inhibition zone only against *V. harveyi* with the inhibition zone 4.4 mm ± 0.6 mm and 3.2 mm ± 0.7 mm on 10000 µg. g^{-1} of extract concentration.

This result is in agreement with the finding of Molina-Cárdenas *et.al* (2014) that demonstrate *Isochrysis galbana* synthesizes antibacterial fatty acids that inhibit the growth of pathogenic bacteria such as *V. harveyi*, *V. alginolyticus,* and *V. campbellii*.

The antimicrobial compounds of microalgae are expected due to their lipids contains. Lipids and some free fatty acids are known to show antibacterial activities from algae (Desbois & Smith, 2010; Plaza *et al.*, 2010). It has been shown that the promoting effect on membrane damage leads to a leakage of molecules from the microbial cells, reduction in nutrient uptake, or inhibition of cellular respiration (Smith *et al.*, 2010). An antibiotically active fatty acid is presented in a high concentration in algae as stated by Hoai Thu *et.al* (2015), found that *I. galbana* Parke, strain HP has the highest content of Docosahexaenoic acid (DHA), up to 14.7% of total fatty acid and maximal Polyunsaturated

fatty acids (PUFAs) values at the early stationary phase. The primary PUFAs are stearidonic acid (18:4n-3) (Nalder *et.al*., 2015).

Conclusion

The crude extract of three species microalgae, *Dunaliella salina*, *Tetraselmis chuii,* and *Isochrysis galbana,* can be used as antibacterial activity against aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence*. The best result for the aquatic purpose was *T. chuii* which can inhibit both pathogenic bacteria *V. harveyi* and *P. fluorescence* and also have antibacterial activities for three replicas. Hence, it is necessary to carry out further research about the availability of microalgae resources as a new functional feed for aquaculture.

Acknowledgments

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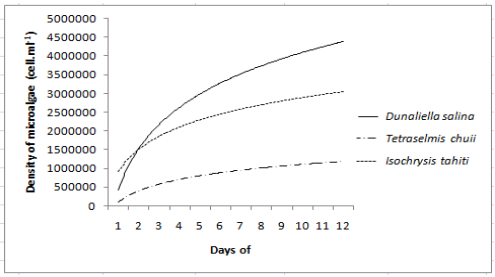

Figure 1: Density of *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* (cell. mL^{-1}) x 10^4.

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Table 1. The biomass of microalgae

No. Species	Wet biomass (g)	Dry biomass (g)
1. *D. salina*	6.65	1.026
2. *T. chuii*	7.97	1.23
3. *I. galbana*	22.65	3.54

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Table 2. The result of crude extract of microalgae with methanol solvent

No.	Species	Dry biomass (*g*)	Extract biomass (*g*)
1.	*D. salina*	1.026	0.33
2.	*T. chuii*	1.230	0.43
3.	*I. galbana*	3.540	1.69

Table 3. Results of McFarland standard for bacteria

No.	Bacteria	OD	Cell density (cell . $mL^{-1)}$
1.	*Vibrio harveyi*	0.671	12 x 10^8
2.	*Pseudomonas fluorescence*	0.107	1.5 x 10^8

Note : OD: Optical Density

Table 4. Antibacterial activity of microalgae against *Vibrio harveyi*

No	Species	Inhibition zone (mm)					
		24 h			48 h		
		a*)	b*)	c*)	a*)	b*)	c*)

1	*Dunaliella salina*	4.4±0.6	1.8±0.4	0	4.3±0.9	1.8±0.3	0
2	*Tetraselmis chuii*	3.5±0.7	2.3±0.3	3.0±0.6	3.5±0.7	2.2±0.3	2.9±0.5
3	*Isochrysis galbana*	3.2±0.7	2.9±0.2	2.5±0.8	3.0±0.6	2.9±0.2	2.5±0.7

Note:
The concentration of *V harveyi* for
a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}
*) Mean ± SD

Table 5. Antibacterial activity of microalgae against *Pseudomonas fluorescence.*

No	Species	Inhibition zone (mm)					
		24 h			48 h		
		a*)	b*)	c*)	a*)	b*)	c*)
1	*Dunaliella salina*	0.0	0.0	0.0	0.0	0.0	0.0
2	*Tetraselmis chuii*[*)]	4.2 ± 1.1	3.2 ± 0.5	3.1 ± 0.1	4.2 ± 0.9	2.9 ± 0.2	3.1 ± 0.2
3	*Isochrysis galbana*	0.0	0.0	0.0	0.0	0.0	0.0

Note:
The concentration of *P. fluorescence* for
a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}
*) Mean ± SD

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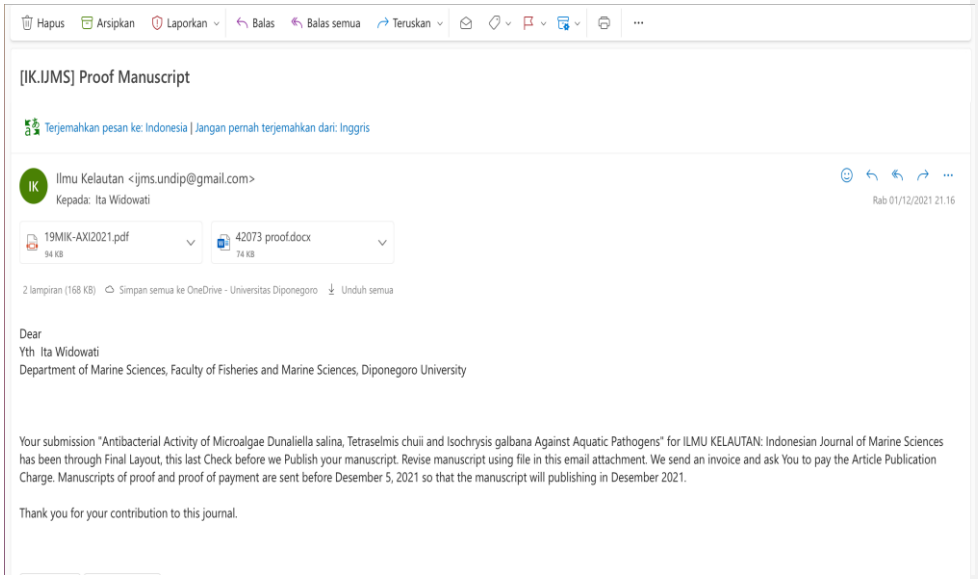

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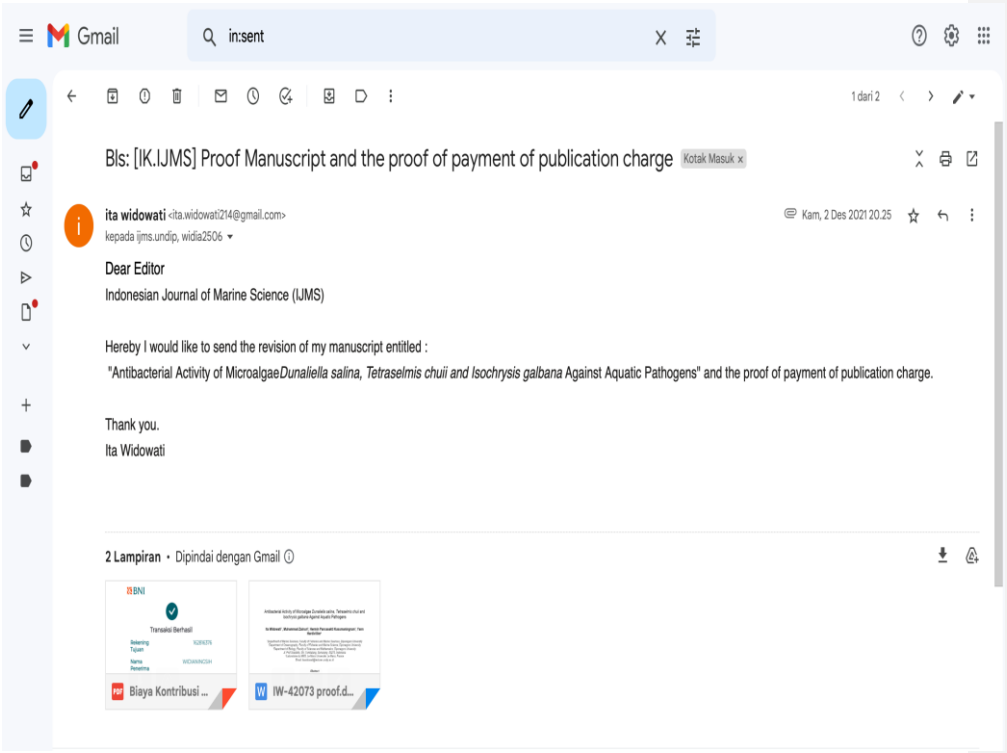

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Antibacterial Activity of Microalgae *Dunaliella salina*, *Tetraselmis chuii* and *Isochrysis galbana* Against Aquatic Pathogens

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Abstract

Recently, expanded consideration has been paid to the business and potentiality of microalgae. Some microalgae are at present being studied for their capacity to find important metabolites for the drug industry or aquacultural applications. Concerning these biotechnological challenges, there is a consistent exertion accommodated in both finding and taking advantage of new microalgal assets and fostering their putative business results or modern valorizations. The crude methanol extracts of three microalgae, Dunaliella salina, Tetraselmis chuii, and Isochrysis galbana, have been investigated for antibacterial activity using the disk diffusion method against aquatic pathogens of fish, shrimp, and shellfish e.g. Pseudomonas fluorescence and Vibrio harveyi. This research aimed to analyze the antibacterial activity of crude extract of these microalgae against aquatic pathogens. The disk diffusion method was used to investigate the antibacterial activity. The result showed that only T. chuii has the inhibition zone for both tested bacteria P. fluorescence and V. harveyi with the inhibition zone of 3.0 ± 0.6 mm on 100 µg. g^{-1} of extract concentration and 4.20 ± 1.1 mm against P. fluorescence on 10000 µg. g^{-1} of extract concentration. While D. salina and I. galbana have inhibition zone only for V. harveyi with the inhibition zone of 4.4 ± 0.6 mm and 3.2 ± 0.7 mm on 10000 µg. g^{-1} of extract concentration. These three microalgae may have potential use in aquatic pathogens as antimicrobial agents. It would be possible to develop biologically active compounds of microalgae as a functional feed for aquaculture.

Keywords: *antibacterial activity, aquatic pathogens, marine microalgae, microalgal extract.*

Introduction

The problems of microbial resistance in aquaculture have led to a search for new antimicrobial compounds. The significant issues of current antimicrobial agents are harmfulness, absence of adequacy, restraining cost, and their frequent use leading to the emergence of resistant strains. Subsequently, there is an earnest need to look for elective biodegradable agents, which should be free from side effects. It is generally that natural compounds are biodegradable and eco-friendly. Microalgae are mainly utilized in aquaculture particularly due to their nutrition contents (Khatoon *et al*, 2014; Hoai Thu *et al*, 2015). Besides nutrition advantages, microalgae also have been explored for the use of pigments and other biological purposes. Different species of microalgae are known as a fundamental food source in the rearing of all stages of marine bivalve mollusks (clams, oysters, and scallops), and of the post-larval stages of some marine gastropods (*e.g.* abalone), larvae of marine fish, penaeid shrimp, and zooplankton. There are three distinct types of live feed, usually utilized in commercial larvae culture of fish and shellfish: 1). Different species of microalgae ranging between 2 µm and 20 µm in size for bivalves, penaeid shrimps, rotifers, copepods, and fish; 2). The rotifers *Brachionus plicatilis* and *B. rotundiformis* (50 to 200 µm) in size for crustaceans and marine fish; 3) The brine shrimp *Artemia* sp. nauplii (400 to 800 µm) in size for crustaceans and fish (Lavens and Sorgeloos, 1996).

The bioactive compound also can be found in macroalgae such as *Sargassum* sp. (antibacterial,

antiviral, antioxidants) (Hardouin *et al.*, 2013; Widowati *et al.*, 2014; Susilowati *et al.*, 2015). However, the application of macroalgae for aquaculture is being limited, for example, it cannot be used as feed for larval stages. Microalgae have rich sources of structurally and biologically active metabolites including antioxidants (Widowati *et al.*, 2017) and antibiotics which inhibit bacteria responsible for fish, shellfish, and human pathogens (Pradhan *et al.*, 2011; Najdenski *et al.*, 2013). Secondary or primary metabolites produced by these microorganisms may be potential bioactive compounds of interest in aquafeed as an antimicrobial agent (Pradhan *et al.*, 2011). Therefore, some microalgae have been suggested as a new functional feed ingredient (Becker, 2004). This research aimed to determine the antibacterial activity of three species microalgae *Dunaliella salina* (Teodoresco, 1905), *Tetraselmis chuii* (Butcher, 1959), and *Isochrysis galbana* against aquatic pathogens *Pseudomonas fluorescence* (Flügge, 1886) and *Vibrio harveyi* (Johnson and Shunk, 1936; Baumann *et al.*, 1981).

Materials and Methods

Culture of microalgae

Three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* was cultured by using batch continuous culture with the comparison between algae and seawater was 1 : 3 L. Walne was used for the nutritional compound for the culture compared by volume of culture 1 mL: 1 L with luminous intensity 1500 lux to 3000 lux and 23 to 25 °C for temperature (Harrison and Berges, 2005).

The density of the culture microalgae was counted with a Neubauer hemocytometer by using a binocular microscope. The density of the microalgae was counted every day. The microalgae were homogenized first before putting the pipet into the hemocytometer and closed with the cover glass. Then, it was observed under a microscope and started to count with the equation (Hadioetomo, 1993):

$$N = \frac{(N1+N2)}{2} x \frac{1}{10.2\,mm^2 \; x \; 0.1mm} x \frac{1mm^3}{10^{-3}\,mL} \quad (1)$$

Note: N = Cell density (cell. mL^{-1}); N1 = Total cell in 80 small squares (replica 1); N2 = Total cell in 80 small squares (replica 2); 0.2 mm = Wide of hemocytometer in 80 squares; 0.1 mm = The depth liquid on a hemocytometer.

The microalgae were harvested on the stationary phase of culture. The biomass of microalgae was obtained with a centrifuge at 5000 rpm (1 rpm equal 1/60 Hz) for 10 min and then dried in a room at view days, then called dry biomass (Mishra *et al.*, 2012).

Extraction

Extraction involves the separation of plant or animal tissues from inactive or inert components by using selective solvents in standard extraction procedures. Dry biomass was extracted by using methanol solvent with sonification at 50 Hz for 15 min. Then, the solvents were evaporated by using rotary evaporation until there is no solvent called with crude extract bioactive of microalgae (Trianto *et al.*, 2011).

Antibacterial activities

The screening of antibacterial activities from extract microalgae against two aquatic pathogens bacteria *Vibrio harveyi* and *Pseudomonas fluorescence* was performed by using the disk diffusion method. The extract of each microalga was diluted into three concentrations *Dunaliella salina* 13.32 µg. g^{-1},1.33 µg. g^{-1}, and 0.13 µg. g^{-1}; *Tetraselmis chuii* 12.61 µg. g^{-1},1.26 µg. g^{-1}, and 0.13 µg. g^{-1}; *Isochrysis galbana* 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}. One of the 50 µL culture of tested bacteria in the logarithmic phase were spread on to agar medium. Several paper disks (8 mm; Advantec Toyo Roshi, Ltd, Japan) containing 30 µL of each concentration were placed on the respective agar surface. The plates were incubated at room temperature for 48 h. Antibacterial activity was defined by inhibition zones around the paper disk (Radjasa *et al.*, 2009).

Results and Discussion

Culture of microalgae

Microalgae were counted for the density every day with a hemocytometer under the microscope binocular. The highest densities are found in *Dunaliella salina* (4.4 x 10^6 cells.mL^{-1}), followed by *Isochrysis galbana* (3.2 x10^6 cells.mL^{-1}), and the lowest is in *Tetraselmis chuii* (0.96 x 10^6 cells.mL^{-1}). However, the highest dry biomass is found in *I. galbana* (3.54 g) and the lowest is in *D. salina* (1.026 g). The highest density in *D. salina* is not in line with the dry biomass, this may be due to the cell size since *D. salina* cell is (2.8-40 µm, Borovkov et.al, 2019) and *I. galbana* cell size is 4-6 µm (Cordoba-Matson, 2013). The cell size could change with growth, light intensity, and different condition (Borowitzka, 2021). The result of the density of microalgae *Dunaliella*

salina, *Tetraselmis chuii,* and *I. galbana* is shown in Figure 1.

Extraction

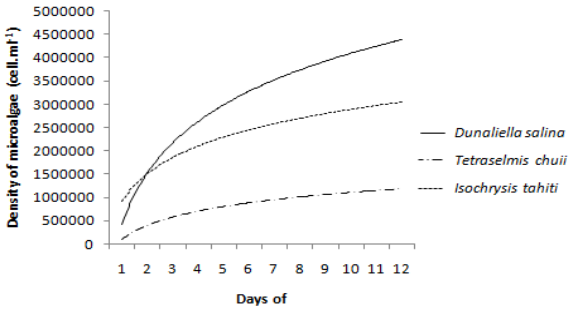

Figure 1. Density of *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* (cell. mL^{-1}) x 10^4.

Table 1. The biomass of microalgae

Species	Wet biomass (g)	Dry biomass (g)
D. salina	6.65	1.026
T. chuii	7.97	1.23
I. galbana	22.65	3.54

Table 2. The result of crude extract of microalgae with methanol solvent

Species	Dry biomass (g)	Extract biomass (g)
D. salina	1.026	0.33
T. chuii	1.230	0.43
I. galbana	3.540	1.69

produce substances inhibiting the growth of selected bacteria was evaluated in this research to support the efforts for fulfilling the important need for aquaculture purposes. Antibacterial activities of three species of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *Isochrysis galbana* against two species of bacteria's aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence* was carried out using the disk diffusion method. The range of the concentrations, *Dunaliella salina* were 13.32 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}; *Tetraselmis chuii* were 12.61 µg. g^{-1}, 1.26 µg. g^{-1}, and 0.13 µg. g^{-1}; and *Isochrysis galbana* were 13.26 µg. g^{-1}, 1.33 µg. g^{-1}, and 0.13 µg. g^{-1}, respectively was conducted for the crude extract from methanol solvent of microalgae. Based on the result, the best concentration will be used for the next purification extracted with another solvent.

Furthermore, the organic polar solvent provided extraction yield, ranging from 0.33 g for *Dunaliella salina*, 0.43 g for *Tetraselmis chuii*, and 1.69 g for *Isochrysis galbana.* It was obtained from 3 L and 1.5 L of culture. The crude extract for methanol solvent with high polarity was able to extract the antimicrobial compound. Several authors (Borowitzka, 1995, Ozdemir *et al*., 2004) have attributed the cyanobacteria antimicrobial activity to different compounds.

Antibacterial activities

The antibacterial activities were carried out by using disk diffusion methods. The tested bacteria were counted for their density by using the McFarland standards method (Table 3). The result of antibacterial activity by using the disk diffusion method against *Vibrio harveyi* is shown in Table 4 and against *Pseudomonas fluorescence* is shown in Table 5. Antibacterial activity of various extracts of the microalgae *Spirulina platensis* (Pradhan *et al*., 2011), *Euglena viridis* (Das *et al.*, 2005), cyanobacteria (Najdenski *et al*., 2013), have been reported as the main groups of microalgae to produce antimicrobial substances.

The result of antibacterial activities in this study showed that only *Tetraselmis chuii* provided the inhibition zone against two tested bacteria *V. harveyi* with the inhibition zone 3.0 mm ± 0.6 mm on 100 µg. g^{-1} of extract concentration and 4.2 mm ± 1.1 mm against *P. fluorescence* on 10 000 µg. g^{-1} of extract concentration. The result is similar to the finding that methanol extracts of *Tetraselmis* sp. showed maximum zone of inhibition against *Pseudomonas* sp (Rajendran, *et al*, 2014). Furthermore, Kokou *et al*

(2012) found that *Tetraselmis chuii* and *Isochrysis* sp showed antibacterial activity against six (6) Vibrio

Table 3. Results of McFarland standard for bacteria

Bacteria	OD	Cell density (cell · mL$^{-1)}$
Vibrio harveyi	0.671	12 x 10^8
Pseudomonas fluorescence	0.107	1.5 x 10^8

Note : OD: Optical Density

Table 4. Antibacterial activity of microalgae against *Vibrio harveyi*

Species	Inhibition zone (mm)					
	24 h			48 h		
	a*)	b*)	c*)	a*)	b*)	c*)
Dunaliella salina	4.4±0.6	1.8±0.4	0	4.3±0.9	1.8±0.3	0
Tetraselmis chuii	3.5±0.7	2.3±0.3	3.0±0.6	3.5±0.7	2.2±0.3	2.9±0.5
Isochrysis galbana	3.2±0.7	2.9±0.2	2.5±0.8	3.0±0.6	2.9±0.2	2.5±0.7

Note: The concentration of *V harveyi* for a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}; *) Mean ± SD

Table 5. Antibacterial activity of microalgae against *Pseudomonas fluorescence*.

Species	Inhibition zone (mm)					
	24 h			48 h		
	a*)	b*)	c*)	a*)	b*)	c*)
Dunaliella salina	0.0	0.0	0.0	0.0	0.0	0.0
*Tetraselmis chuii**)	4.2 ± 1.1	3.2 ± 0.5	3.1 ± 0.1	4.2 ± 0.9	2.9 ± 0.2	3.1 ± 0.2
Isochrysis galbana	0.0	0.0	0.0	0.0	0.0	0.0

Note: The concentration of *P. fluorescence* for a: 10 000 µg. g^{-1}; b: 1000 µg. g^{-1}; c: 100 µg. g^{-1}; *) Mean ± SD

bacterial strains: *V. parahaemolyticus, V. anguillarum, V. splendidus, V. scophthalmi, V. alginolyticus,* and *V. lentus*. Besides antibacterial activity, *Tetraselmis chui* is an important producer of fatty acid, and this is an insignificant relationship with environmental conditions (Mohammadi *et al.*, 2015).

Meanwhile, two microalgae, *Dunaliella salina* and *Isochrysis galbana* tested, have the inhibition zone only against *V. harveyi* with the inhibition zone 4.4 mm ± 0.6 mm and 3.2 mm ± 0.7 mm on 10000 µg. g^{-1} of extract concentration. This result is in agreement with the finding of Molina-Cárdenas *et.al* (2014) that demonstrate *Isochrysis galbana* synthesizes antibacterial fatty acids that inhibit the growth of pathogenic bacteria such as *V. harveyi*, *V. alginolyticus,* and *V. campbellii*.

The antimicrobial compounds of microalgae are expected due to their lipids contains. Lipids and some free fatty acids are known to show antibacterial activities from algae (Desbois and Smith, 2010; Plaza *et al.*, 2010). It has been shown that the promoting effect on membrane damage leads to a leakage of molecules from the microbial cells, reduction in nutrient uptake, or inhibition of cellular respiration (Smith *et al.*, 2010). An antibiotically active fatty acid is presented in a high concentration in algae as stated by Hoai Thu *et.al* (2015), found that *I. galbana* Parke, strain HP has the highest content of Docosahexaenoic acid (DHA), up to 14.7% of total fatty acid and maximal Polyunsaturated fatty acids (PUFAs) values at the early stationary phase. The primary PUFAs are stearidonic acid (18:4n-3) (Nalder *et.al*., 2015).

Conclusion

The crude extract of three species microalgae, *Dunaliella salina*, *Tetraselmis chuii,* and *Isochrysis galbana,* can be used as antibacterial activity against aquatic pathogens *Vibrio harveyi* and *Pseudomonas fluorescence*. The best result for the aquatic purpose was *T. chuii* which can inhibit both pathogenic bacteria *V. harveyi* and *P. fluorescence* and also have antibacterial activities for three replicas. Hence, it is necessary to carry out further research about the availability of microalgae resources as a new functional feed for aquaculture.

Acknowledgments

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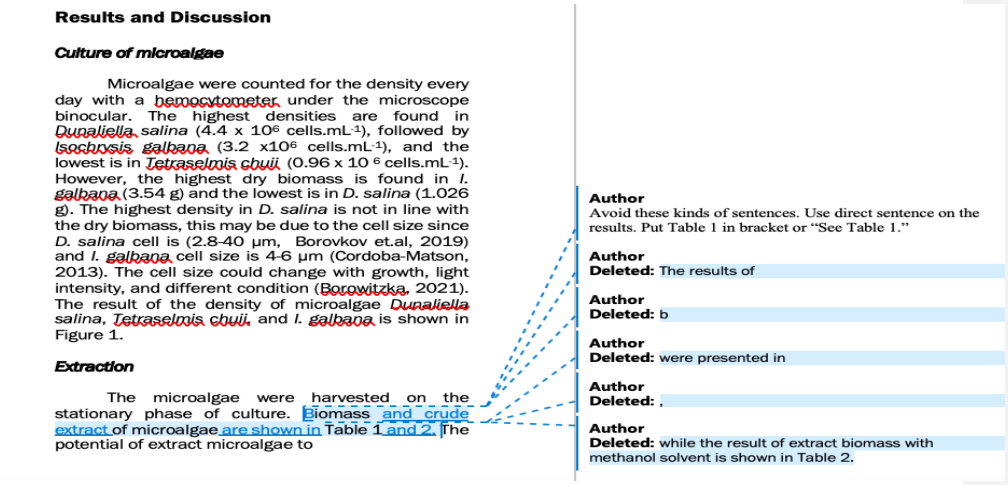

Results and Discussion

Culture of microalgae

Microalgae were counted for the density every day with a hemocytometer under the microscope binocular. The highest densities are found in *Dunaliella salina* (4.4 x 10^6 cells.mL^{-1}), followed by *Isochrysis galbana* (3.2 x10^6 cells.mL^{-1}), and the lowest is in *Tetraselmis chuii* (0.96 x 10^6 cells.mL^{-1}). However, the highest dry biomass is found in *I. galbana* (3.54 g) and the lowest is in *D. salina* (1.026 g). The highest density in *D. salina* is not in line with the dry biomass, this may be due to the cell size since *D. salina* cell is (2.8-40 µm, Borovkov et.al, 2019) and *I. galbana* cell size is 4-6 µm (Cordoba-Matson, 2013). The cell size could change with growth, light intensity, and different condition (Borowitzka, 2021). The result of the density of microalgae *Dunaliella salina*, *Tetraselmis chuii*, and *I. galbana* is shown in Figure 1.

Extraction

The microalgae were harvested on the stationary phase of culture. Biomass and crude extract of microalgae are shown in Table 1 and 2. The potential of extract microalgae to

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