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The Effect of Oxidative Stress on Growth and Antioxidant Activity of Microalgae *Oocystis sp*

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Abstract

This study was conducted to see the effect of growth and antioxidant activity of microalgae *Oocystis sp* before and after adding NaOCl to Growmore medium combined with urea. Antioxidant activity test was conducted by calculating IC_{50} spectrophotometrically with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. *Oocystis sp* extract was obtained by sonication and maceration process with methanol solvent. The results showed the best growth curve for the growth of microalgae *Oocystis sp* in a mixture of Growmore medium 0.02% b/v and Urea 0.002% b/v. These results also showed a decrease in *Oocystis sp* growth in a mixture of Growmore medium 0.02% b/v and Urea 0.002% b/v with the lowest growth decrease in the addition of NaOCl with a concentration of 0.01%. The IC_{50} value of *Oocystis sp* extract in methanol solvent has high antioxidant activity in Growmore 0.02% b/v + Urea 0.002% b/v medium, which is 55.5862 mg/L. It can be concluded that NaOCl affects the growth and antioxidant activity of microalgae *Oocystis sp*, but at certain concentrations NaOCl experienced a negative effect.

Keywords: Microalgae, Oocytis sp, Oxidative Stress, Growth, Antioxidant

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Abstrak (Indonesian)

Penelitian dilakukan untuk melihat pengaruh pertumbuhan dan aktivitas antioksidan mikroalga *Oocystis sp* sebelum dan sesudah ditambahkan NaOCl kedalam medium Growmore yang dikombinasikan dengan urea. Untuk uji aktivitas antioksidan atau perhitungan IC_{50}, penelitian ini menggunakan metode 1,1-*diphenyl*-2-*picrylhydrazyl* (DPPH) secara spektrofotometri. Sedangkan untuk mendapatkan ekstrak *Oocystis sp*, dilakukan dengan proses sonikasi dan maserasi dengan pelarut metanol. Hasil penelitian menunjukkan kurva pertumbuhan terbaik untuk *Oocystis sp* pada campuran medium growmore 0,02% b/v dan urea 0,002% b/v. Hasil juga menunjukkan penurunan pertumbuhan *Oocystis sp* pada campuran medium Growmore 0,02% b/v dan Urea 0,002% b/v dengan penurunan paling rendah pada penambahan NaOCl dengan konsentrasi 0,01%. Nilai IC_{50} ekstrak *Oocystis sp* pada pelarut metanol memiliki aktivitas antioksidan yang tinggi pada medium Growmore 0,02% b/v + Urea 0,002% b/v yaitu 55,5862 mg/L. NaOCl berpengaruh terhadap pertumbuhan dan aktivitas antioksidan mikroalga *Oocystis sp*, namun pada konsentrasi tertentu NaOCl dapat berpengaruh negatif.

Kata Kunci: Mikroalga, Oocytis sp, Stres Oksidatif, Kurva Pertumbuhan, Antioksidan

INTRODUCTION

Antioxidants are compounds that can inhibit oxidation reactions by binding free radicals to form unreactive molecules [1]. Antioxidants have an important role in maintaining health [2] . This is due to the ability of oxygen to inhibit free radicals. Antioxidants are widely used as additives in the food industry to prevent fat oxidation. Such antioxidants are increasingly commercialized as nutraceuticals and food supplements [3].

Natural antioxidant sources are mostly found in plants. One of which is in microalgae. Microalgae are more consider being a new source of natural antioxidants. Most microalgae contain carotenoid antioxidants, tocopherols (vitamin E), ascorbic acid (vitamin C) or phenolic compounds [4]. In this study, the source of natural antioxidants used was the microalgae *Oocystis sp*. One of the problems encountered in research on microalgae is the small amount of biomass obtained from the culture results, therefore Growmore fertilizer is combined with Urea fertilizer ($CO(NH_2)_2$) for the growth of microalgae *Oocystis sp*. This is done because Urea fertilizer is easy to obtain and the nitrogen content reaches 46%

Nitrogen is the main component of protein formation which is very much needed for cell multiplication. Some efforts have been made to increase antioxidant activity with various oxidative stresses. In the study of [5] it has been proven that oxidative stress with the addition of NaOCl can trigger increased antioxidant activity in microalgae *Nannochloropsis oculata*, *tetraselmis sp* and *Closterium ehrenbergii* [5]. The general form of oxidative stress given to microalgae is in the form of high salinity, light, temperature, and nutrient content [6]

Appart from mentioned above, this is still necessary to conduct the effect of stress on other microalgae because the ability to adapt to stress in microalgae varies [7]. Based on this, the cultivation of *Oocystis sp* microalgae was carried out in a mixture of Growmore medium and urea added with NaOCl to stimulate secondary metabolites in microalgae. NaOCl will form hypochlorous acid (HOCl) when it reacts with water which will be able to disrupt protein folding and inhibit glucose oxidation, causing biochemical damage to *Oocystis sp* microalgae cells. Microalgae can accumulate various amounts of metabolites under different nutrient stress conditions (either nutrient-enrichment or nutrient deficient). In other words, the nutrient stress is one strategy to boost the accumulation of a given targeted metabolite in the microalgae cells. The selection of nutrient conditions is influenced by well-studied mechanisms of metabolite hyperaccumulation [8,9].

The biocidal effect of NaOCl also involves the action of free radicals that cause oxidative stress to produce ROS (reactive oxygen species) which disrupts microalgae metabolism. Increased ROS levels have the potential to harm microalgae cells causing oxidation of lipids, proteins, DNA and ultimately cell death. Nevertheless, cells are equipped with good antioxidant defense mechanisms to detoxify the effects of ROS [10]. The biomass obtained was extracted using the maceration method using methanol as a solvent. The antioxidant activity testing process in this study used DPPH (1,1-diphenyl¬-2-picrylhydrazyl) reagent as a source of free radicals. Therefore, this study needs to be conducted since observing the effect of growth and antioxidant activity of microalgae *Oocystis sp* before and after adding NaOCl as well as detecting antioxidant activity with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.

MATERIALS AND METHODS

Materials and Instruments

The equipment used in this study include: UV-VIS spectrophotometer Thermo Scientific Genesys 20, centrifuge, oven, autoclave, freezer, Light Microscope, Hotplate, magnetic bar, Analytical balance, Aluminum foil. The materials used in this study include: Oocystis sp which has been isolated by Wahida Nia elfiza in the Biochemistry laboratory of Andalas University, Padang, Growmore fertilizer, Urea fertilizer, Methanol (Merck), Ascorbic Acid (Merck), (Merck) Diphenyl Picryl Hydrazil (DPPH) (Merck), aquades, plastic Wrap.

Identification of microalgae morphology Oocystis sp

Microalgae isolates obtained from the Biochemistry Laboratory of Andalas University were observed for their morphology using a light microscope with a magnification of 1000x. Then the morphology obtained was matched with the morphology of *Oocystis sp* in the literature.

Making media growmore + urea fertilizer media

The medium was made in several stages. The first stage was making Growmore 32 medium with a concentration of 0.02%. Growmore medium was made by weighing 0.2 g of Growmore fertilizer dissolved using 1L of aquadest. The second stage was the urea solution made by weighing 1 g of urea and dissolving it in 100 mL of aquadest. The third stage was the Growmore 32 solution mixed with urea solution in 300 mL of medium. The urea variations used were 0.002% w/v, 0.004% w/v, 0.008% w/v, and 0.0012% w/v of the

Growmore concentration in the medium taken from a 1% urea solution, with a total volume of 0.02% w/v Growmore medium and 0.002% w/v urea of 300 mL.

Addition of NaOCl

Growmore medium with the best urea fertilizer variation was added with NaOCl variations, namely 0.002%; 0.004%; 0.006%; 0.008%; 0.01% with an additional volume of 0.05 mL; 0.1 mL; 0.15 mL; 0.2 mL; and 0.25 mL of 12% NaOCl in 300 mL of medium. NaOCl was added to the stationary phase (day 16).

Cultivation of Oocystis sp microalgae

Oocystis sp microalgae were cultured in BBM, Growmore, Growmore + urea medium in 500 mL bottles under the same conditions. The seeds used were 1:10 of the culture volume. Every day the growth of the culture was observed by measuring the Optical Density (OD) value using a UV-VIS spectrophotometer at a wavelength of 400 nm.

Preparation of Oocystis sp microalgae wxtract

Oocystis sp microalgae were first harvested in the stationary phase. The culture was harvested using the flocculation method (microalgae sedimentation) by turning off the microalgae aeration and leaving it alone. The precipitate formed was transferred into a petri dish and air-dried to obtain dry biomass. Then, 0.2 g of dry biomass was extracted using 10 mL methanol, the extraction process was assisted by using a sonicator at a frequency of 480 Hz for 1 hour and then macerated for one day. Each extract was combined and then dried until a collection of extracts was obtained.

Antioxidant activity test with DPPH method

The DPPH solution was made by dissolving 10 mg of DPPH in a 250 mL measuring flask with methanol to the boundary mark. The solution was protected from sunlight and incubated in the dark for 30 minutes at room temperature. As a positive control, 5 mg of ascorbic acid was used, dissolved with methanol in a 10 mL measuring flask to the boundary mark, obtaining a concentration of 500 mg / L. Then 4 variations of concentrations were made 10; 15; 20; and 25 mg / L by the dilution method. On the other hand, the test solution was made by dissolving 5 mg of extract with methanol in a 10 mL measuring flask to the boundary mark, obtaining a concentration of 500 mg / L. Then 4 variations of concentrations were made 10; 15; 20; and 25 mg/L with dilution method. Each test solution and positive control were taken as much as 2 mL then added 3 mL of DPPH solution and left for 30 minutes in a dark place. As a negative control in this test is 2 mL of methanol plus 3 mL of DPPH solution and as a blank used is 5 mL of methanol. Then the absorbance of each concentration of test solution and control was measured at a wavelength of 517 nm. Based on the absorbance obtained, the % inhibition was calculated by the formula:

$$\text{Inhibition} = \frac{\text{Control Absorbant -Sample Absorbant}}{\text{Control Absorbant}} \text{ x } 100\% \quad (1)$$

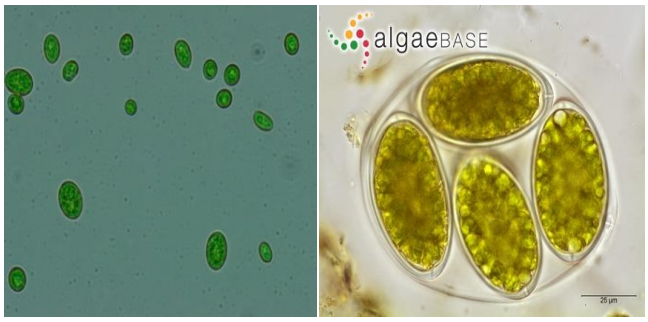
Information:
Control absorbance: DPPH + Methanol
Sample absorbance: DPPH + Methanol + sample

To determine the IC_{50}, plot the sample concentration and percent inhibition on the x and y axes of the linear regression equation. The equation is used to determine the IC_{50} of each sample expressed by the value of y = 50 and the value of x to be obtained as the IC_{50}. Criteria for Assessment of Antioxidant Activity [11].

RESULTS AND DISCUSSION

Identification of microalgae morphology

The results of the morphological check obtained were compared with the morphology of *Oocystis sp* literature to find out whether the microalgae used were single isolates. If the microalgae used are contaminated, it can result in data errors during the antioxidant activity test later, because it is not only the extract from *Oocystis sp* that is measured. The identification process of *Oocystis sp* microalgae is carried out before being propagated and harvested because it is to ensure whether what is being studied is the microalgae *Oocystis sp* before proceeding to the further testing stage.



(a) (b)

Figure 1. (a) Morphology of *Oocystis sp*. results of observation of magnification 1000x and (b) morphology of microalgae *Oocystis naegelii*

Results of morphological identification of microalgae *Oocystis sp* using a light microscope depicted on **Figure** 1.a. In **Figures** 1.a and 1.b, it can be seen that there are similarities in the shape of microalgae *Oocystis sp* namely both ends of the cell are

concave (pointed) and the cell wall is thick, surrounded by stem cells but sometimes unicellular, ellipsoidal body cells, single pyrenoid chloroplasts[12]. From the identification that has been carried out, it can be seen that the microalgae used are a single culture of *Oocystis sp*.

Growth of Oocystis sp microalgae culture

Growth of microalgae culture before adding NaOCl

The results of observations conducted for 20 days showed that *Oocystis sp* cultured in the medium as a whole formed the same growth, including the lag phase, exponential phase, stationary phase, and death phase and had different cell densities every day. The lag phase occurs on days 0-2 where cells adapt to the culture medium given nutrients. This phase is characterized by changes in cell size shortly after inoculation into the medium. However, division has not occurred so that cell density has not increased and the population has not changed [13]. Furthermore, the exponential phase occurs on days 3-10. The exponential phase is characterized by a period of rapid growth, cells divide at a constant rate, metabolic activity is constant, and a state of balanced growth between food supply and microalgae increase [14]. Then the stationary phase on days 11-16, in this phase the growth rate is slower compared to the exponential phase, in this phase there is an accumulation of toxins due to microalgae metabolism, lack of nutrients, and changes in environmental conditions [15,16]. So that there is a breakdown of primary metabolites into secondary metabolites, resulting Oocystis sp relies on secondary metabolites to grow. Next is the death phase, in this phase the number of dead microalgae cells is greater than the living cells and the food reserves in the cell body are reduced [7].

Cell growth is indicated by the increasing concentration of the culture color. From figure 2 it can be seen that the best position for microalgae growth is in a mixture of Growmore 0.02% b/v and Urea 0.002% b/v medium. The highest absorbance peak was obtained in Growmore 0.02% b/v and Urea 0.002% b/v medium which indicates the large number of microalgae cells growing in the medium. The growth of microalgae in other media is not much different from Growmore 0.02% b/v and Urea 0.002% b/v medium. This is because the amount of urea in the medium is not much different, namely urea 0.002% b/v as much as 0.6 mL, urea 0.004% b/v as much as 1.2 mL, urea 0.008% b/v as much as 2.4 mL, and urea 0.0012% b/v as much as 3.6 mL. The image also shows the lowest growth of *Oocystis sp* microalgae in BBM medium, this is because the nutrient content in BBM medium is less suitable for the growth of microalgae *Oocystis sp*.

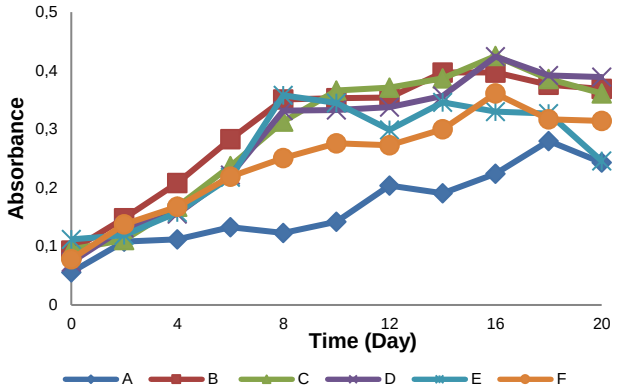


Figure 2. Growth curve of *Oocystis sp* microalgae on medium: (A) BBM, (B) Growmore, (C) Growmore 0.02% b/v + urea 0.002% b/v, (D) Growmore 0.02% b/v + urea 0.004% b/v, (E) Growmore 0.02% b/v + urea 0.008% b/v, (F) Growmore 0.02% b/v + urea 0.0012% b/v.

Culture growth after addition of NaOCl

The culture process with the condition of Growmore medium 0.02% b/v and Urea 0.002% b/v until the stationary phase (before induction) lasted for 16 days. When reaching the stationary phase, the induction process was carried out for 3 days (days 17-20) with the addition of NaOCl 0.002, 0.004, 0.006, 0.008, 0.01%, respectively. From each of these concentration variations, there is a change in the growth rate of *Oocystis sp* which has an impact on microalgae production.

In **Figure** 3, growth without the addition of NaOCl, namely Growmore 0.02% b/v and Urea 0.002% b/v is better than that added with NaOCl. It can be seen that after adding NaOCl, there was a decrease in the absorbance value of the measured culture. At NaOCl concentrations of 0.01% and 0.008%, there was a greater decrease in growth than at NaOCl concentrations of 0.002%, 0.004%, and 0.006%. So it can be concluded from Figure 3 that the more NaOCl is added, the more the growth of *Oocystis sp* microalgae decreases. This is because NaOCl will form hypochlorous acid (HCl) when it reacts with water which will disrupt protein folding and inhibit glucose oxidation, causing biochemical damage to *Oocystis sp* microalgae cells. Therefore, the growth of *Oocystis sp* microalgae is inhibited [17].

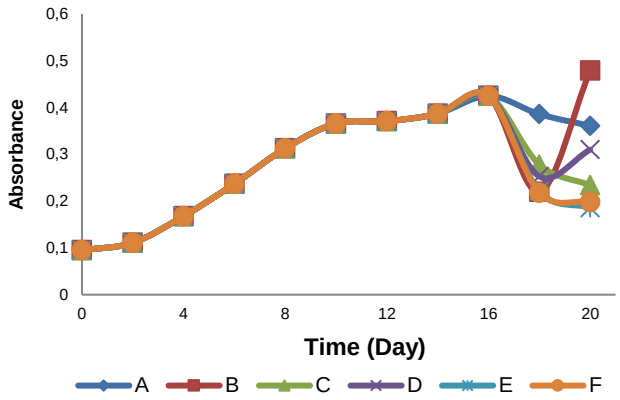


Figure 3. Growth curve of *Oocystis sp* microalgae on Growmore 0.02% b/v + urea 0.002% b/v medium given NaOCl: (A) without NaOCl, (B) 0.002%, (C) 0.004%, (D) 0.006%, (E) 0.008%, (F) 0.01%.

It can be concluded in **Figure** 3 that the tendency for microalgae growth after the addition of NaOCl decreased on the 17th to 20th day. This is thought to be caused by the ammonium concentration in the medium not being sufficient for the needs of *Oocystis sp* microalgae to support the growth of *Oocystis sp* cells [11] and the concentration of NaOCl added was large enough so that the microalgae could not survive in the stationary phase. This results in the growth of *Oocystis sp* cells being inhibited so that the cells enter the death phase where many microalgae cells die than live.

Extraction of Oocystis sp

Oocystis sp microalgae were harvested in the stationary phase. This was done because in the stationary phase there was an accumulation of toxins due to microalgae metabolism, lack of nutrients, and changes in environmental conditions [18]. This causes secondary metabolites in microalgae to form a lot to protect themselves from free radical attacks. So that microalgae can survive. Then the microalgae were extracted using a polar methanol solvent to dissolve polar compounds [19].

Antioxidant activity test

The relation between antioxidant activity and the concentration of Growmore is shown in Figure 4. From the Figure 4, the percentage of inhibition of *Oocystis sp* extract at a concentration of 25 mg/L in a mixture of medium (A) Growmore (GM) 0.02% b/v containing 0.002% b/v urea, and Growmore (GM) 0.02% b/v containing 0.002% b/v urea with variations in the addition of NaOCl (B) 0.002%, (C) 0.004%, (D) 0.006%, (E) 0.008% and (F) 0.01%, respectively, are 45.5279%, 44.0484%; 44.8554%; 44.2502%; 44.8554%; and 44.8554%, respectively.

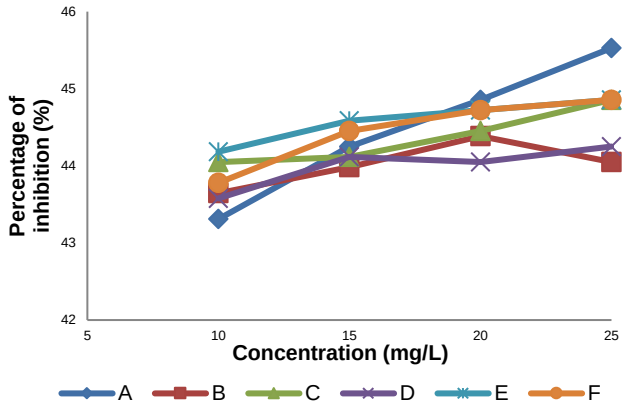


Figure 4. Inhibition percentage of *Oocystis sp* microalgae extract on Growmore medium 0.02% w/v + urea 0.002% w/v given NaOCl: (A) without NaOCl, (B) 0.002%, (C) 0.004%, (D) 0.006%, (E) 0.008%, (F) 0.01%.

Based on **Figure** 4, it can be seen that the greater the concentration of *Oocystis sp* extract, the greater the inhibition percentage. This is because the more active compounds that capture free radicals in solution to capture free radicals in DPPH so that the remaining free radicals are getting smaller. The inhibition percentage is used to determine the inhibition percentage of *Oocystis sp* extract against free radical compounds.

Meanwhile, based on the IC50 value, the Growmore (GM) sample 0.02% b/v containing 0.002% b/v urea with variations in the addition of NaOCl 0.002% has the highest value as seen in Figure 5.

Based on **Figure** 5 the lowest IC_{50} value of microalgae extract occurred in medium A, which was 55.5862 mg/L. This shows that the *Oocystis sp* extract in medium A is a strong antioxidant because its IC_{50} value is between 50-100 ppm. Medium B is a weak antioxidant because its IC_{50} value is greater than 200 ppm. Medium C is a moderate antioxidant. Medium D is a weak antioxidant. Medium E is a moderate antioxidant. And medium F is a strong antioxidant. Ascorbic acid is used as a comparison which has a very active antioxidant activity, which is 0.2694 mg/L.

In the results of this study, it can be concluded from Figure 5 that with the addition of NaOCl with a concentration of 0.002%; 0.004%; 0.006%; 0.008%; and 0.01% antioxidant activity did not increase. Meanwhile, NaOCl is suitable as oxidative stress to produce ROS by increasing the percentage of antioxidant activity of *Cyanobacterium*, *Tetra Selmis sp*, and *Anabaena sp* microalgae with NaOCl concentrations used of 0.5, 10, and 50 µM [20].

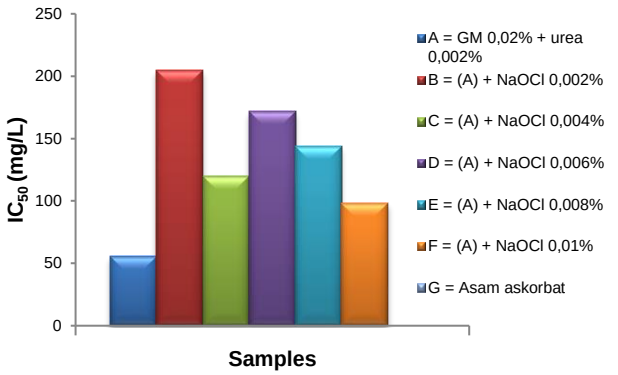


Figure 5. IC_{50} value of *Oocystis sp* microalgae extract

Based on this, it can be concluded that the cause of NaOCl not being able to stimulate antioxidants in *Oocystis sp* microalgae because the concentration of NaOCl added was large enough so that *Oocystis sp* microalgae did not have time to produce secondary metabolites to maintain themselves in the stationary phase. This resulted in faster death of *Oocystis sp* microalgae cells which was marked by a decrease in the microalgae growth curve. Hand in hand with the research from Dharma [21] in the study stated that to neutralize excess reactive oxygen species (ROS) with antioxidant compounds, this can be done with various types of microalgae such as *Chlorella Vulgaris, Scenedesmus Ellipsoidal, Scenedesmus Dimorphous, and Oocystis Sp.*

CONCLUSION

Based on the research, the following conclusions can be noted are that the best composition of Growmore and Urea mixture for the growth of *Oocystis sp* microalgae is a mixture of Growmore 0.02% b/v and urea 0.002% b/v. NaOCl affects the growth and antioxidant activity of *Oocystis sp* microalgae. In this case, the effect obtained is negative. It is hoped that this study can provide information with high NaOCl concentrations that cannot trigger an increase in antioxidant activity in *Oocystis sp* microalgae. *Oocystis sp* extract in methanol solvent has high antioxidant activity in a mixture of Growmore 0.02% b/v and urea 0.002% b/v with an IC_{50} value of 55.5862 mg/L.

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