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Improvement of the potency of microalgae *Nannochloropsis* and *Chaetoceros* through antioxidant analysis and optimization of DNA isolation

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Abstract. *Nannochloropsis* and *Chaetoceros* are microalgae that commonly used as natural feed for aquaculture animals. These microalgae are able to produce secondary metabolites that is used as an antioxidant. The objective of the study was to analyze the antioxidant activity from both microalgae using the DPPH method in relation with their DNA quality and quantity. The method for DNA isolation optimization was Doyle and Doyle method with slight modification. The results of this research showed the percentage of inhibition of *Nannochloropsis* and *Chaetoceros* were 9.72 % and 5.78%, respectively. While optimization of DNA isolation exhibited that *Nannochloropsis* was 193,5 ng/ul with a purity of 1,99 while *Chaetoceros* was 93.9 ng / μ l with a purity of 2.00.

1. Introduction

Nannochloropsis and *Chaetoceros* are the member of diatoms (class Bacillariophyceae) which serve as promising sources of sustainable antioxidants due to their ability as effective radical scavengers [1]. As natural feed in water environment, they have the ability to adapt and rapidly grow *Nannochloropsis* is a uniselluler greenish microalgae[2]. The cells are ball shaped, spherical or slightly ovoid. *Nannochloropsis* has 2-4 micron cell size, elongated round shape, has two flagella (heterocontous), one of which has thin haired flagella, has a chloroplast which has a stigma that is sensitive to light and contains chlorophyll a and c[2]. It is also harbor fucoxanthin and violaxanthin pigments with one chloroplast in each cell. *Nannochloropsis* was cosmopolitan microalgae and able to grow at 0-35 ppt salinity with optimum salinity 25-35 ppt in marine and freshwater. Optimum temperature for growth is 25-30°C and grows well in the pH range of 8-9.5. Metabolite composition of *Nannochloropsis* consist of protein 55.80–80%, carbohydrate 16–20.10%, lipid 11.00–17.00%, EPA 2.50%, DHA 1.80%, chlorophyll A 0.89%, vitamin C 0.85%, water content 3.60 % and ash content of 4.50% [3-6].

Chaetoceros sp. belong to diatom group (*Bacillariophyceae*). This diatom is the important phytoplankton and contribute dominantly to marine productivity, especially in coastal waters.



Chaetoceros sp. perform asexual reproduction by cell division and sexual by the formation of auxospores. Cell division in phytoplankton will produce two daughter cells. A daughter cell that receives an epithelial part will develop to resemble the size of its parent cell, while a daughter cell that receives a hypothemic part will grow smaller than a stem cell. *Chaetoceros* sp. Was widely used as natural feed because besides having a high enough protein content, in suitable environmental conditions, the density of this natural food is rapidly increasing. Nutritional content of *Chaetoceros* sp. is 35% protein, 6.9% fat, 6.6% carbohydrate and 28% ash content [7–11].

Chaetoceros and *Nannochloropsis* have been suspected of having strong antioxidant properties. An antioxidant is a compound that at low concentrations can significantly inhibit or prevent oxidation of the substrate in a chain reaction. Antioxidants can protect cells from damage caused by unstable molecules known as free radicals. Antioxidants can donate electrons to free radical molecules, so they can stabilize free radicals and stop chain reactions. Examples of antioxidants include β -carotene, lycopene, vitamin C, vitamin E. Antioxidants can be grouped into two parts, namely primary or natural antioxidants and secondary or synthetic antioxidants. Antioxidants are substances that can prevent or inhibit the oxidation process so that they form more stable compounds. Antioxidants polyphenols are the group most abundant in fruits, vegetables, and plants. Synthetic antioxidant compounds have the function of capturing free radicals and stop chain reactions. Several examples synthetic antioxidants are Butylated hydroxyl anisole (BHA), Butylated hydroxytoluene (BHT), Propyl gallate (PG) and metal chelating agent (EDTA), Tertiary butyl hydroquinone (TBHQ), Nordihydro guaretic acid (NDGA) [1,10,11].

One method used to determine antioxidant activity is the DPPH (2-2-Diphenyl-1-Picrylhydrazyl) method. The DPPH method is a method that is often chosen as a method of testing antioxidant activity because it is simple, easy, fast, sensitive and requires a small sample. This method only requires DPPH compounds that are stable and comparative compounds such as vitamin A, vitamin C and vitamin E. In addition, this method does not require a substrate because free radicals are already available directly to replace the substrate [6].

Microalgae have a complex and unique cell wall structures which often hinders the extraction of their genetic material to meet the required quality and quantity. The difficulties of the DNA isolation from eukaryotic microalgae has repeatedly been reported [12-15] and this has been attributed to their unique cell wall structures, The DNA isolation is done with the aim to separate DNA from other materials such as proteins, fats, and carbohydrates. *Nannochloropsis* and *Chaetoceros* are microalgae that have cell walls that are much different. DNA isolation from both microalgae using the same isolation method will be an interesting study to see its impact on the quality and quantity of DNA obtained. The cell wall of *Nannochloropsis* sp. was made from strong cellulose components part of a complex carbohydrate that can bind toxic substances. *Nannochloropsis* sp. is a type of green cell algae that can be used to adsorb metal ions. The adsorption ability of *Nannochloropsis* sp. quite high because in the algae *Nannochloropsis* sp. there are functional groups of amines, amides and carboxylates that can bind to metal ions [7]. This diatom contains a single cell or a series of cells with a hard outer part and area skeleton-silica layer (pectin containing silica) called frustule [5]. Most of the protocols currently used for DNA extraction from photosynthesis microalgae are essentially the same as those applied for plants. The previous research stated that although the standar CTAB method or generally known as Doyle and Doyle method was very useful and robust for plant species, it produce lower amounts of microalgae DNA, which is not effective and can not be carried out for all species of microalgae[16]. The research objective was performed DNA isolation on *Chaetoceros* and *Nannochloropsis* to separate DNA from other materials such as proteins, fats, and carbohydrates. The main principles in this method is cell lysis, extraction of DNA, and DNA purification. Doyle & Doyle's method is a method of DNA isolation that commonly used for isolation of plant DNA containing polysaccharides and polyphenol compounds. This method used CTAB buffer (Cetyl Trimethyl Ammonium Bromide) [16].

2. Materials and methods

2.1. Materials

The materials used in the study were *Nannochloropsis* and *Chaetoceros* from Balai Besar Penelitian dan Pengembangan Budidaya Laut Gondol Bali Indonesia

2.2. DNA isolation

DNA isolation was conducted according to Doyle and Doyle method [16] with slight modification. The modification was performed with the use of polyvinylpyrrolidone (PVP) 0.01 gr; Isopropanol 2 µl; Sodium acetate (Na-Acetate) 3M 80 ml [17]. It was eliminate the use of mercaptoethanol, ammonium acetate, RNase and Sodium chloride in Doyle and Doyle method. DNA isolation using the Doyle & Doyle method using buffer CTAB (Cethyl Trimethyl Ammonium Bromide) 2%, 70% ethanol, CIA (Chloroform-Isoamyl-Alcohol), and Tris EDTA buffer.

2.3. Analisis of quality and quantity of DNA

The DNA was measured using NanoDrop Software (ND-2000) at wavelength 260 nm, 280 nm. The qualitative and quantitative test serves to measure the concentration and purity of DNA.

2.4. DPPH antioxidant analysis

The antioxidant activity of microalgae ethanol extract was tested based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) [18,19]. A total of 10 mg of sample was dissolved in PA methanol then a series of concentrations of 10000 ppm was made. A total of 160 µl extracts from each series of sample concentrations, plus 40 µl DPPH solution. After 30 minutes at room temperature, absorbance was measured at a wavelength of 517 nm. As a negative control used methanol with the same workmanship and as a blank used 200 µl methanol p.a. without adding DPPH solution. Percent inhibition is calculated by the formula:

$$\% \text{ Inhibition} = \frac{(A-B)-(C-D)}{(A-D)} \times 100\%$$

Explanation: A = Negative control absorbance
 B = Blank Absorbance
 C = Extract control absorbance
 D = Extract absorbance

The degree of colour change of the solution from purple to yellow indicates the efficiency of free radical scavenger followed by incubation the solution for 30 minutes. Inhibition percentage data is used to find the Inhibition Concentration 50 value in ppm. IC₅₀ values were determined by regression analysis.

3. Result and discussion

Microalgae are in the basic position of the food chain in aquatic ecosystems where they use H₂O and CO₂ to synthesize complex organic compounds with the help of sunlight which produce many primary and secondary metabolites as antioxidants. Furthermore, the microalgae showed adaptive response to oxidative stress, through stimulation of their antioxidant defense systems using enzymatic and non-enzymatic mechanisms. Microalgae use their antioxidant capacity as deoxyribose and DNA protection [18]. A reactive oxygen species (ROS) will react with DNA and concomitantly produce strand breaks. The oxidative stress in microalgae was a harmful process that can lead to cell death through oxidation of protein, lipid, and DNA [20]. Main component in microalgae consist of lipids, nucleic acids (RNA and DNA), and proteins represent the main targets of ROS, reactive nitrogen species (RNS), and reactive sulfur species (RSS) [21].

3.1. Antioxidant activity of *Nannochloropsis* and *Chaetoceros*

High antioxidant concentration will make the conversion from the purple chromogen radical (DPPH) to the pale yellow hydrazone. Antioxidant activity of *Nannochloropsis* and *Chaetoceros* was showed on Table 1. The IC₅₀ is the concentration of the sample to inhibit 50% of free radicals. Extract of

microalgae is said to have very strong antioxidant activity if their IC_{50} value is less than 50 ppm. The antioxidant activity will strong if the IC_{50} value is 50–100 ppm. It said moderate if it is 100–150 ppm and weak if the IC_{50} value is 150–200 ppm [18,19]. The result showed that the antioxidant activity of *Nannochloropsis* was lower comparing with *Chaetoceros* sp although both microalgae showed strong activity of antioxidant. These antioxidants may influence their gene expression associated with biotic and abiotic stress responses to maximize defense in their environment [3,10,22].

Table 1. Antioxidant activity of *Nannochloropsis* and *Chaetoceros*

Microalgae	Concentration	%Inhibition	IC_{50} Value
<i>Nannochloropsis</i>	10.000	5.78	72.368
<i>Chaetoceros</i>	10.000	9.72	61.760

Previous study with *C. calcitrans* showed lower value of IC_{50} about 3.83 $\mu\text{g/mL}$ under the same solvent [23]. *N. oculata* showed higher percentage of inhibition value of 21.68% [21]. Many phenolic compounds in microalgae have been reported as antioxidants, but they might not be major contributors to the antioxidant capacities of microalgae since they also have natural antioxidant pigments (carotenoids and chlorophylls), fucoxanthin and PUFAs that can be found on some microalgae including *C. calcitrans* [2,10,23–26]. *Nannochloropsis* sp. also showed relatively lower total phenolic content than the extract of *Chaetoceros* sp [3,8,24] which is supported our result.

3.2. DNA isolation of *Nannochloropsis* and *Chaetoceros*

The results of DNA isolation for *Nannochloropsis* and *Chaetoceros* was showed on Table 2. The results exhibited that *Nannochloropsis* and *Chaetoceros* had gained good quality of purification.

Table 2. Quality and Quantity of DNA *Nannochloropsis* dan *Chaetoceros*

Microalgae	DNA Concentration ng/mL	Purity (A_{260}/A_{280})
<i>Nannochloropsis</i>	1.99	193.5 ng/ml
<i>Chaetoceros</i>	2.00	93.9 ng/ml

The value of DNA purity ranges from 1.8–2.0 which is indicated that both DNA of microalgae have no contamination of protein groups, phenol compounds, and RNA. The result was supported by another researcher in acquiring good result on quality DNA isolation for microalgae [12,13,15]. The concentration of DNA achieved lower value for *Chaetoceros* comparing with *Nannochloropsis* as showed in Table 3. Although the values were less than commonly DNA concentration on plants using the same method, both microalgae gaining higher concentration comparing with other researcher. The yield of DNA were still adequate for most uses in further step of molecular biology techniques and the concentration of DNA was above average with other studies. Isolation DNA of *Prototheca wickerhamii* yielded low DNA concentrations from $\text{ng}/\mu\text{L}$ 16.15 into 74.2 $\text{ng}/\mu\text{L}$ followed by purity below 1.8 and above 2.0 indicating existence of residual phenol or carbohydrate carry over [12].

Since our research was conducted slight modification for Doyle and Doyle method with the addition of PVP, isopropanol and sodium acetate, it seem that this modification was suitable and effective in gaining more DNA comparing with standart method, which was in agreement with previous study [17]. Its also exhibited that modification Doyle and Doyle method was suitable for *Nannochloropsis* and *Chaetoceros* to gain high quality and quantity of DNA. The good method for DNA isolation for microalgae must be a combination of high concentration and high purity of DNA, with no evidence of contamination from cellulose, polysaccharides, proteins or RNA. Furthermore the method must not change the structure and function of DNA molecules; and the method must be simple, low cost and efficient [15,16].

Since adaptive response to oxidative stress in microalgae was conducted through induction of their antioxidant defense systems, microalgae will use their antioxidant capacity to protect their DNA [6,20,22]. Consequently, the more antioxidants produced by microalgae will decrease the amount of DNA due to oxidation stress. This process is thought to occur in *Chaetoceros* which obtained lower concentration of DNA compared to *Nannochloropsis*. This results is very interesting and need to be improved for further research.

4. Conclusion

In the present study, an efficient and reliable procedure of *Nannochloropsis* and *Chaetoceros* DNA isolation extraction was gaining high quality and quantity of DNA. The quantity of DNA isolation was higher for *Nannochloropsis* than *Chaetoceros* which is correlating with higher antioxidant concentration that reduce DNA. The modification of Doyle and Doyle method performed here represents a considerable improvement over the standart methods of DNA isolation for the cell-walled eukaryotes microalgae.

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