Description of *Chlorella pyrenoidosa* Isolated from Pathek Beach, Situbondo Regency, Indonesia

Elya Putri Pane[a], Yenny Risjani[b*], Yunianta[c], Gilang Drajat Maulana[a], Nurul Mutmainnah[b]

Abstract
Waters are habitats for biodiversity, one of which is microalgae. Microalga is a very interesting water microorganism because it has yet to be expanded into further utilization. This microorganism is confirmed to have protein, carbohydrates, lipids, pigments, and other bioactive compounds considered the third-generation raw material for biofuel production. Microalgae biomass can be utilized to support the fulfillment of functional food. As with the diversity available in water, exploration research is carried out to obtain pure isolates to be utilized. Microalgae samples were taken at Pathek Beach (East Java Province), Situbondo Regency with the horizontal column method. This study aims to analyze the pure isolated strains and identified morphology, analyzing metabolic components such as protein and total lipid. The research results include total protein analyzed by the lowry-follin method. The total lipid content is analyzed by the soxhlet method. Chlorella pyrenoidosa contains a total protein content of 41.556 mg/l and a lipid content of 5,05%.

Keywords: *Chlorella pyrenoidosa*, Pathek beach, morphology, isolation, protein, lipid

[a]Master's Degree Program in the Faculty of Fisheries and Marine Science, University of Brawijaya, Veteran Street, 65145, Malang City, Indonesia
[b*]Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Veteran Street, 65145, Malang City, Indonesia
[c]Department of Agricultural Technology, Faculty of Agricultural Technology, University of Brawijaya, Veteran Street, 65145, Malang City, Indonesia

***Corresponding Author: -** email: risjani@ub.ac.id

Introduction

Indonesia is an archipelago with 25.000 islands spread from Sabang to Merauke. The water area is a habitat for biodiversity (Andréfouët *et al.,* 2022; Lasabuda, 2013), which is a unit of various ecosystems on earth, including macroalgae (Risjani & Abidin, 2020) and microalgae (Arsad *et al.,* 2022; Risjani *et al.,* 2021). Microalgae are aquatic microorganisms containing proteins, carbohydrates, lipids, pigments, and other bioactive compounds (Lafarga, 2020), considered third-generation raw materials for biofuel production (Duran *et al.,* 2018). The main groups of microalgae are Bacillariophyceae, Chlorophyceae, Cyanophyceae, and Chrysophyceae (Ghayal & Pandya, 2013).

Chlorella pyrenoidosa is classified into green microalgae *(Chlorophyceae)* with high biotechnology potential (Garrido-Cardenas *et al.,* 2018). Chlorophyceae has high adaptability to various environmental conditions and high productivity in cultivation systems because the benefits contained therein can be utilized for various fields of biotechnology (Baldisserotto *et al.,* 2022; Mathur *et al.,* 2021). This microorganism has a spherical cell shape with a size of 2-10 µm and contains chlorophyll (Daliry *et al.,* 2017). The cell structure is simple and requires light, water, and nutrients to grow properly (Lu *et al.,* 2019). The culture medium must have sufficient nutrients for optimal microalgae growth. The components essential to support microalgae growth include the elements C, N, P, S, Fe, and Mg. The composition contained in the microalgae media also affects the target microalgae (Zeng *et al.,* 2021).

The abundance of microalgae in Pathek Beach, Situbondo, Indonesia, has yet to be fully exploited. The richness abundance is due to the lack of identification activities from microalgae. Through isolation to the pure stage of microalgae isolates from the results of sampling in these waters, identification of *Chlorella pyrenoidosa* isolates morphologically and screening for protein, and total fat content can be identified.

Material & Methods Material:

This research started by isolating samples from Pathek Beach, Situbondo Regency, Indonesia. Furthermore, morphological isolation and identification of *Chlorella pyrenoidosa* were carried out in June – December 2022. Then the pure culture was carried out to obtain biomass so that protein and total lipid content could be analyzed properly. In addition, other parameters observed were the growth rate and parameters of the quality of the *Chlorella pyrenoidosa* culture media. The research material used includes research tools and materials.

The equipments used included test tubes, 5 falcons 50 ml, erlenmeyer 250 – 1000 ml, glass pasteur pipette, bunsen, aerator, tubular lamp, hemocytometer, petri dish, centrifuge, and autoclave. The materials include sterile seawater, 70% alcohol, distilled water, vitamin B12, and nutrients. The nutrients used were F/2 Walne with a composition of 1 liter of distilled water containing 45 g of EDTA, 100 g of $NaNO_3$, 0,36 g of MnCl, 33,6 g of H_3BO_3, 20 g of NaH_2PO_4 and 1,3 g of FeCl3.

Location And Time of Research:

The research was conducted from April to December 2022. The location for sampling was taken at Pathek Beach, Situbondo Regency, East Java Province, Indonesia, with coordinates 7°38'33.89"S and 113°59'21.36"E. Sample isolation was carried out at the hydrobiology laboratory of the fish resources division of Brawijaya University, Scanning Electron Microscope (SEM) analysis was carried out at the Materials Laboratory, testing for protein content was carried out at the Chemistry Laboratory at Malang State University, and testing for total lipid content was carried out at Airlangga University.

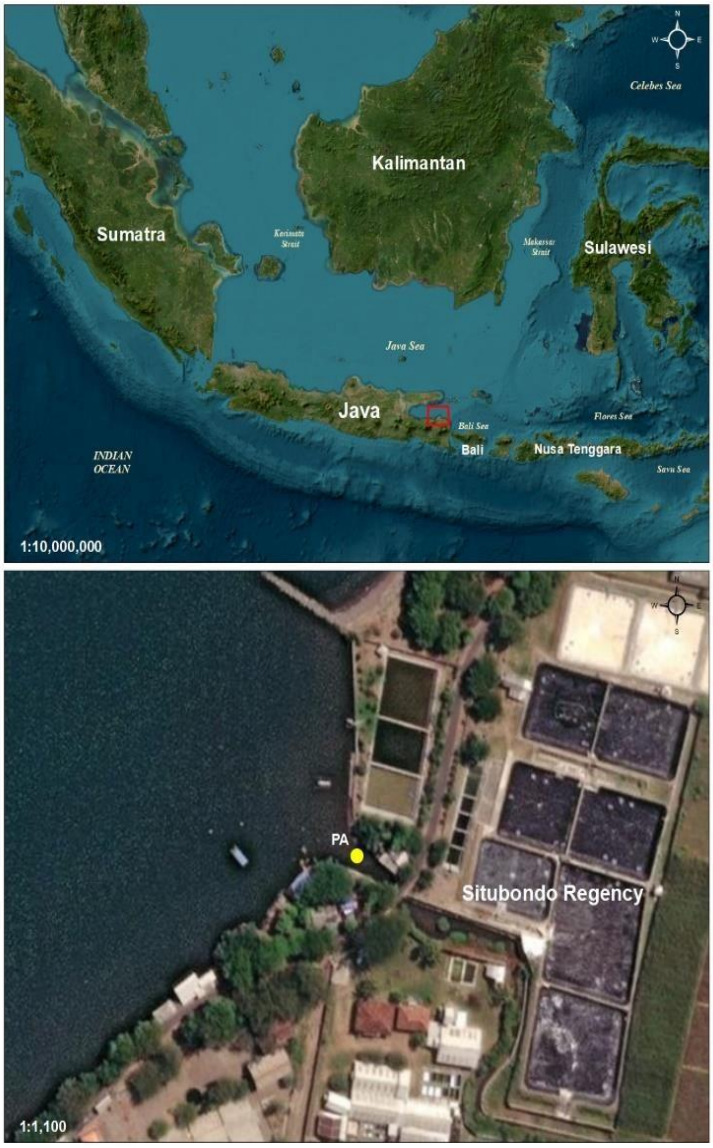

Fig 1: Sampling site of *Chlorella pyrenoidosa* in Pathek Beach, Situbondo Regency, East Java, Indonesia

Sampling and Isolation:

Sampling was carried out in this study by modification according to Nasution *et al.* (2019) vertical and horizontal methods in the water column which can be done using a plankton net mesh size of 20 µm in the water column and 50 ml of the falcon (Rahmah *et al.,* 2022). Isolation was carried out in this research using the pipetting method. The prepared sample then poured ± 5 ml into the petri dish placed on the microscope. The equipments prepared include an inverted microscope Olympus IX 53 with a minimum magnification of 400x and uses a glass pipette for isolation. The capillary glass pasteur pipette was heated with bunsen and then pulled with tweezers to form a small diameter.

Pure Cultivation:

A pure culture was carried out after selected success at the isolation stage. Before the pure culture stage was conducted, the equipments must be sterilized first to avoid unwanted microorganisms (contaminants) used for microalgae cultivation. The equipment used, such as a test tube, erlenmeyer, and aeration hose, immersed in a chlorine solution at a dose of 1 ml for 1 liter of water. Then, the soaking time was waiting for 24 hours. The aeration hose was inserted into the carboy containing chlorine solution for sterilization. Then, rinsed with clean water and dried (Buwono & Nurhasanah, 2018). In addition, seawater that has been prepared must also be sterilized with

chlorine and neutralized with a solution of Sodium Thiosulfate. The medium used in this study was seawater which had been sterilized.

Density Calculation, Specific Growth Rate, and Doubling Time:

Microalgae growth was observed by calculating the number of cell densities daily using a hemocytometer to determine the peak density. Then it was rinsed with alcohol 70%, and the sample was dripped with a volume of 1 ml. Then covered with a cover glass and observed under a microscope with a magnification of 40 times (Cahya *et al.,* 2020). The following is the microalgae density formula (Erdawati *et al.,* 2020):

$$D = \{\frac{N}{2} \quad \frac{}{n} 1+N2 \; x \; 25 \; x \; 10^4 \}$$

Descriptions:

D = Density (cells/mL)

N = Number of cells

10^4 = Total actual cell density in 1 ml of media

The specific growth rate was calculated from the growth at the start of the culture to the death phase. To find out the specific growth rate (K') can be calculated by the formula (Moheimani *et al.,* 2012):

$$K' = \frac{ln(Nt0 - Nt1)}{t}$$

Descriptions:

K' = Growth rate (/day)

Nt_0 and Nt_1 = Initial density at the first time and density at the second time (mL) t = Time interval (days)

Cell doubling time (dt) is the average cell generation time. The doubling time can be calculated using the following formula (Ak *et al.,* 2008):

$$dt = \frac{ln\ 2}{\mu}$$

Total Protein Analysis:

Protein was analyzed using the Lowry-Follin method according to Slocombe *et al.* (2013); Fakhri *et al.* (2020) with several reagents. Determination of protein in the sample dissolved 0,25 g of material in distilled water to a volume of 100 ml, then filtered. Next, put 1 ml of the sample into a test tube, then add 1 ml of the reagent that has been prepared, shake it immediately until it is homogeneous, and incubate at room temperature for 15 minutes. Add another 3 ml of reagent to the sample tube and shake as soon as possible, then incubate at room temperature for 45 minutes and immediately measure the absorbance at 550 nm. The blue color formed remained stable for 45-80 minutes after incubation. The measurement results are plotted into the regression equation of the standard solution that has been made so that the protein concentration is known.

Total Lipid Analysis:

Pargiyanti (2019) analyzed total lipid using the soxhlet method with the following procedure. 2 g sample put in filter paper covered with cotton. The filter paper containing the sample was plugged with cotton and dried in an oven at a temperature not exceeding 80°C for 1 hour. The filter paper containing the dried sample was put into the soxhlet apparatus. The Soxhlet apparatus was connected to a lipid flask containing boiling stones that had been dried, and the weight is known. The extracted sample was done with petroleum ether solvent for 6 hours. Petroleum ether was distilled, and the fat extract was dried in an oven at 105°C, then cooled and measured to a constant weight. The results obtained were calculated using the following formula:

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

Scanning Electron Microscope (SEM):

The sample to be analyzed was placed in a holder measuring ±10 mm. The sample used for testing uses Au-Pd coating to ensure that the sample could be more conducive. The sample was put into the SEM Chamber and then pumped, and after it was thoroughly vacuumed, the SEM machine was ready for use (Beam On).

Results and Discussion

Morphology of *Chlorella pyrenoidosa*:

SEM analysis results showed that successfully isolated isolates could be seen morphologically showing *Chlorella pyrenoidosa* can be seen in Figure 2. *Chlorella sp.,* green microalga, have a round body shape like an egg, ball, or oval forms. In Figure 2, it is explained that the morphology of the microalgae has a spherical

shape with a cell diameter ranging from 2 – 8 µm. This microalga does not have flagella, so it cannot move actively (move slowly) and has a cell wall consisting of cellulose and pectin. Each *Chlorella sp.,* have a cell nucleus and chloroplast. An illustration of the morphology of *Chlorella pyrenoidosa* can also be seen in figure 3 (Shihira & Krauss, 1963; Safi *et al.,* 2014). The morphology of the microalgae isolated from Pathek Beach was collected from the harvested biomass after the fourth day of cultivation. The harvesting phase have fourth day. The harvested biomass was centrifuged at 2500 rpm for 10 minutes and then dried in an oven for 18 hours.

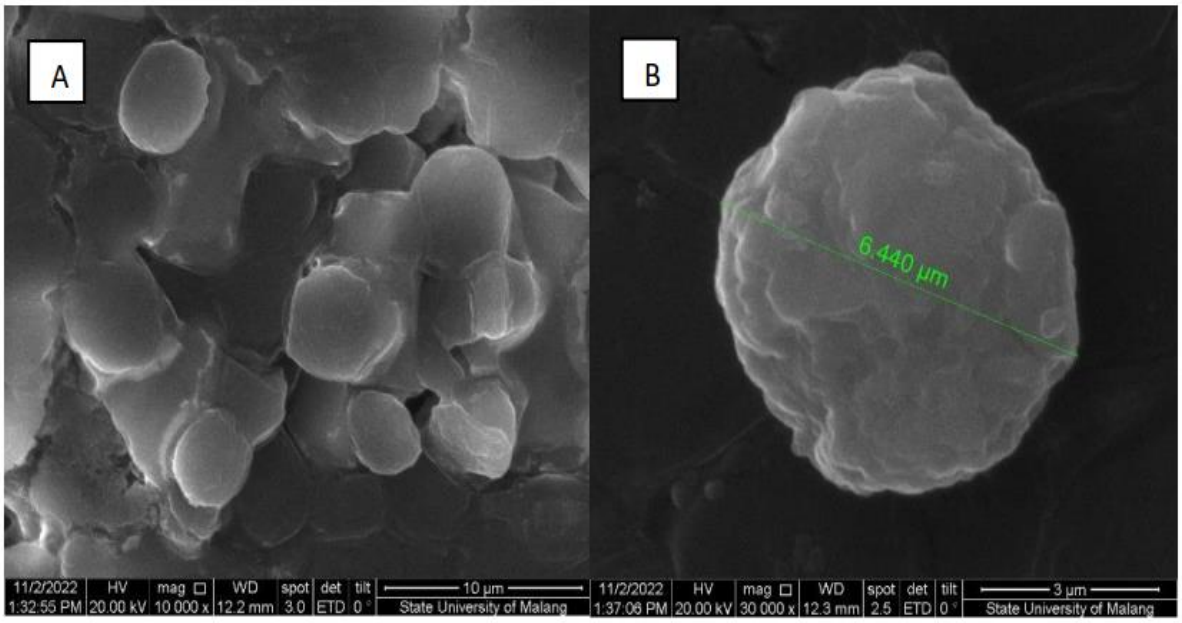

Fig 2: Morphology of *Chlorella pyrenoidosa* isolate with SEM isolated from Pathek Beach, Situbondo Regency, East Java Province

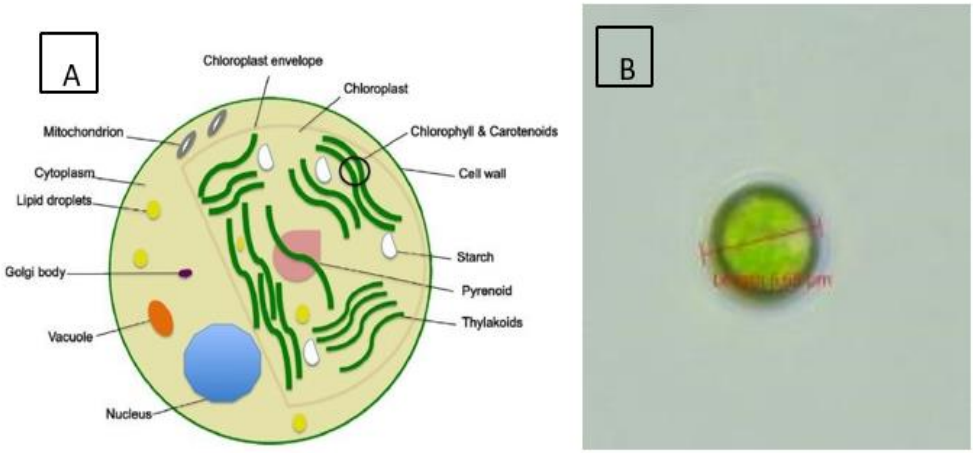

Fig. 3: A) Ilustration of *Chlorella sp.* cell organelles (Safi *et al.,* 2014) B) Light Microscope of this study with Olympus CX21 Microscope (magnification 400 times)

Growth Rate and Doubling Time: of 1 ml/L resulted in a growth rate of The research conducted for 10 days (Table 1) *Chlorella pyrenoidosa* of 0,69027427 and a showed that giving Walne fertilizer at a dose doubling time with a value of 1,00416.

Table 1: Specific growth and doubling time of this study

Species Name	Specific Growth (/days)	*Doubling Time*
Chlorella pyrenoidosa	0,69027427	1,00416

The specific growth rate is directly proportional to the cell density; the higher the cell density, the higher the growth rate (Nurhanifah *et al.,* 2018). The growth rate is a parameter that describes the rate of increase of microalgae isolate cells per unit of time. According to Sorokin & Krauss (1959), culture conditions can affect the growth phase of microalgae so that they can cause changes in the composition of the media. Abdurrachman *et al.* (2013), an optimal growth rate will result in optimal productivity. Applying environmental stress, such as modification of nutrients, light intensity, salinity, pH, and temperature, can affect nutrients' productivity and microalgae growth. Meanwhile, according to Nurhanifah *et al.* (2018), doubling time is the time when doubling the cell mass of microalgae isolates double the initial amount. The quick doubling time enters the exponential phase in which the cells divide rapidly and constantly. Each microalga has a different doubling time, and the specific growth rate is inversely proportional to the doubling time.

Quality Parameters of Cultivation Media: The isolate *Chlorella pyrenoidosa* is successfully pure cultivated to determine the culture media's temperature, pH, nitrate, and phosphate parameters.

Table 1. Quality parameters of cultivation media

Species	Parameters			
	Suhu (^{o}C)	pH	Nitrat (mg/L)	Fosfat (mg/L)
Chlorella pyrenoidosa	24,3 - 25,4	7,64 - 7,84	0,5387 - 1,1451	1,569 - 4,9065

Based on the observation results, the culture medium temperature of *Chlorella pyrenoidosa* at the laboratory scale showed results that were still at the optimum for the growth of the isolate. The temperature of the isolated culture is 24.3 - 25.4^{0}C, which is still within the optimum limit for microalgae growth. The optimal temperature for the growth of some microalgae ranges from 20 - 30^{0}C (Khan *et al.,* 2018). Furthermore, the results of pH measurement on microalgae isolate culture media were obtained, which ranged from 7,64 to 7,98. The pH is within the optimum limit for microalgae growth. The optimal pH for microalgae culture is 7 - 9 (Imelda *et al.,* 2018). Based on the results of nitrate measurements, namely 0,5387 – 1,6724 mg/l. The optimal nitrate level needed to support microalgae growth is 0,9 – 3,5 ppm. The results of the phosphate measurements utilized by the two microalgae isolates were 1,569 – 4,9065 mg/l. Phosphate is one of the nutrients needed for the growth of microalgae reproductive activities (Rosyadi *et al.,* 2022). Amini & Syamdidi (2016) state that optimal phosphate levels for microalgae growth range from 0,27 -5,51 mg/l.

Total Protein of *Chlorella pyrenoidosa*:
The protein content of *Chlorella pyrenoidosa* was obtained based on the research containing around 41,555 mg/L. The nitrogen content in the microalgae culture medium affects the protein content. Nitrogen is needed as a macronutrient to support the growth and density of microalgae. Research such as differences in nutrient composition can affect the composition of lipid, protein, and carbohydrates. Microalgae will form protein when the nutrients in the cultivation media are sufficient, and measurements are usually taken when entering the exponential phase. If there is a shortage of macronutrients, microalgae cells will experience a decrease in protein content followed by degradation of other cell components related to protein synthesis (Fery *et al.,* 2020).
Chlorella vulgaris cultured in BG11 media and a nitrogen source from urea contains a protein content of 536,3 mg/L, equivalent to 41,3%. *Chlorella sirokiniana* cultured in the same medium, namely BG11, had a protein content of 588 mg/L or equivalent to a protein content of 25,7% on the third day of cultivation. These two microalgae species were isolated from southern Taiwan's fresh waters (Lai *et al.,* 2019). In addition, *Chlorella pyrenoidosa* contains 57,3% crude protein (Angiotensin *et al.,* 2021) and peptides that can be used as antioxidants, anticancer and anti-inflammatory. The protein content is in the form of dry powder

obtained from Qingdao, China, by spray drying. Both types of green microalgae have been confirmed to contain nutrients that can be used as ingredients for making supplements (Silva *et al.,* 2019).

Total Lipid of *Chlorella pyrenoidosa*:

Chlorella pyrenoidosa isolate contained 5,05% total lipid on pure isolate cultures without any treatment. Nutrients F/2 Walne and vitamin B12 are given at 1 ml/L dose. The total lipid in microalgae was affected by culture conditions such as modification on the light intensity, carbon dioxide, temperature, pH, and nutrition (Zullaikah *et al.,* 2019). Nitrogen is an essential macronutrient that is also needed for growth and lipid metabolism (Fakhry & Maghraby, 2015). Furthermore, the total lipid content in *Chlorella vulgaris*, obtained by Jawa *et al.* (2014), was 8.4514.37% in pure isolate culture. In general, the lipid content in microalgae is usually in the form of glycerol and fatty acids with chain lengths C14 to C22, which can be found in saturated and unsaturated forms. *Chlorella sp.* lipid extraction, which was carried out using the soxhletation method, obtained 12,8818%. This value is slightly affected by the presence of water content in dry biomass, which is worth 9.4% (Kurnia *et al.,* 2018). The total lipid of *Nannochloris atomus* obtained from the Korea Marine Microalgae Culture Center reaches 30-4%. The nutrient given to this pure culture is F/2 Guillard. The method used for total lipid analysis was based on Bligh and Dyer (1959), using modified methanol and chloroform solvents (Kim *et al.,* 2021). Hawrot-Paw *et al.* (2021) also used the same method, and the results showed that the total lipid content of *Chlorella vulgaris* and *Chlorella fusca* was 13% and 14%, respectively, with a nutrient dose of 100%.

Conclusions

Morphological identification of *Chlorella pyrenoidosa* by Scanning Electron Microscopy and light microscope, isolated from Pathek Beach, Situbondo Regency, East Java, Indonesia, contained a protein content of 41.555 mg/L and a total lipid content contained 5,05%.

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