**Food Research 8 (6) : 85 - 95 (December 2024)**
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

RESEARCH PAPER

# Isolation and antiradical activity of phycobiliprotein compounds from *Chlorella pyrinoidosa*

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***Article history:***
Received: 18 December 2022
Received in revised form: 19 January 2023
Accepted: 28 February 2024
Available Online: 20 November 2024

***Keywords:***
*Chlorella pyrinoidosa,*
Isolation,
Antiradical activity,
Phycobiliprotein

***DOI:***
https://doi.org/10.26656/fr.2017.8(6).524

**Abstract**

*Chlorella pyrinoidosa* is known to have the highest protein content compared to other microalgae. One of the proteins which has the potential to be developed as a food supplement is phycobiliprotein. The objective of this study was to isolate and determine the antiradical activity of phycobiliprotein compounds in *Chlorella pyrinoidosa*. The extraction process was carried out using the freeze-thawing method. Aqua pro injection solvent was added to the microalgae biomass, and the mixture was vortexed for 20 s. The pellets obtained were frozen for 24 hrs at -4°C and then thawed at 25°C. After the sample melted, the separation process was carried out by centrifugation at a speed of 3,500 rpm for 15 mins and purified using a cellulose membrane dialysis tube with weight molecular cut-off 12-14 kDa for 12 hrs at 4°C. The supernatant was then freeze-dried. The phycobiliprotein was characterized by Fourier Transform Infrared (FTIR) spectrophotometer to determine the functional groups, SEM (Scanning Electron Microscopy) to determine the topography of the surface of the specimen, and the ninhydrin test to qualitatively prove the presence of amino acids or proteins in a sample. *Chlorella pyrinoidosa* contained phycobiliprotein compounds with the characteristics of having the functional groups of O-H, N-H and C=O, the shape of tiny granules that were unevenly distributed on the surface, and some cracks and clusters which were quite diverse, with an average diameter of 10 µm. Phycobiliprotein in *Chlorella pyrinoidos*a had moderate antiradical activity with an $IC_{50}$ value of 141.254 ppm using the 2,2-Azinobis(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) method. Because of its antioxidant activity, the phycobiliprotein compound in *Chlorella pyrinoidosa* has the potential to be used as a food supplement.

## 1. Introduction

Bioactive compounds in microalgae have quite diverse amounts and variations. Microalgae consist of chlorophyll, carotenoids, and phycobiliproteins. They have antiradical activity and can be used as natural dyes. However, phycobiliprotein is not as widely studied as chlorophyll and carotenoids (Karseno *et al.,* 2013). Phycobiliprotein is a pigment-protein complex with a bright color, water-soluble, and works as a light catcher to assist in the ongoing process of photosynthesis. Green phycobiliproteins are classified into three groups: phycocyanin (blue pigment), allophycocyanin (blue pigment), and phycoerythrin (red pigment) (Su *et al.,* 2014). This pigment is known to have acted as an anticancer, has antimicrobial, antiradical, and anti-inflammatory properties, and can be used as a natural dye (De Morais *et al.,* 2018). A study shows that the antiradical properties of phycobiliproteins work by inhibiting neuronal death through free radical adjustment mechanisms (Hidhayati *et al.,* 2020). Recently, the potential of phycobiliprotein as a photosensitizer that plays a role in photodynamic therapy in the treatment of cancer has started to be recognized. In the pharmaceutical world, phycobiliprotein can be used as a nutraceutical (Karseno *et al.,* 2013). The many benefits of phycobiliproteins have encouraged quite a lot of research to develop these benefits. Several biochemical industries that have started selling phycobiliproteins include Seta Biochemical, Invitrogen, and Folgen. The price of food-grade phycocyanin powder is 1-5 US$/g, while pure phycocyanin powder can reach 250 US$/5 mg or 10-50 US$/mg (Kissoudi *et al.,* 2018).

Based on previous research, there are several types of microalgae containing phycobiliprotein pigments,

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eISSN: 2550-2166 
Published by Rynnye Lyan Resources

including *Chlorella vulgaris* (Djunaedi *et al.,* 2017), *Cystomonad* (Begum *et al.,* 2016), *Porfira* sp. (Osório *et al.,* 2020) and *Gelidium pulsilium* (Mittal *et al.,* 2019).

Natural flavors and colors are generally healthier than artificial ones. Natural sources have their own nutritional and medical qualities, such as antiradical properties (Sorour *et al.,* 2014). One study found that antiradical properties of phycobiliproteins work by inhibiting neuronal death (Hidhayati *et al.,* 2020). The antiradical activity of phycobiliproteins, which is evaluated using the DPPH method from the microalgae *Spirulina* sp., is categorized as weak with an $IC_{50}$ value of 110.80 ppm (Ridlo *et al.,* 2016), while the antiradical activity of the microalgae *Chlorella vulgaris* is strong with an $IC_{50}$ value of 38.900 ppm (Novianti *et al.,* 2019). This can be influenced by the high percentage of protein in the microalgae. The percentage of protein in *Spirulina platensis* is 46% (Nur, 2014), and in *Chlorella vulgaris* 51% (Nur, 2014; Happy *et al.,* 2019). *Chlorella pyrinoidosa* has higher protein content (reaching 57%) compared to several microalgae such as *Anabaena cylindria* (43%), *Chlamydomonas rheinhard* (48%), *Spirulina platensis* (46%) (Nur, 2014), *Chlamydomonas rheinhardii* (48%), *Porphyridium cruentum* (39%), and *Euglena gramaculis* (39%) (Nur, 2014; Happy *et al.,* 2019). The protein levels in *Chlorella pyrinoidosa are* expected to have significant amounts of phycobiliproteins with strong antiradical activity.

Antiradicals are compounds that can reduce the detrimental effects of oxidative stress and reactive oxygen (ROS) (Sonani *et al.,* 2015). Increased ROS and oxidative stress can cause various chronic diseases, such as cancer, diabetes, and heart disease (Hidhayati *et al.,* 2020). Therefore, antiradical properties are required to inhibit free radicals and to protect the body from the dangers of oxidative damage. Antiradicals are divided into two types: synthetic antiradicals such as BHA (Butylated Hydroxyl Anisole) and BHT (Butylated Hydroxyl Toluene), and natural antiradicals such as phycobiliproteins (Hidhayati *et al.,* 2020). Antiradical activities of phycobiliproteins have been demonstrated by both *in vivo* (Guzmán-Gómez *et al.,* 2018) and *in silico* (Sonani *et al.,* 2015) methods.

Considering the above discussion, the objective of this study was to investigate the isolation and the antiradical activity of phycobiliprotein from the microalga *Chlorella pyrinoidosa* using the ABTS method. Because this method is more sensitive, it can be used in a wide range of pH, and compounds that are soluble in water and organic solvents.

## 2. Materials and methods

### *2.1 Manufacture of artificial seawater and nutrition making*

A total of 1 L of ASW was made by weighing 24.6 g of NaCl, 0.6 g of KCl, 1.36 g of $CaCl_2$, 6.2 g of $MgSO_4$, 4.66 g of $MgCl_2$, and 0.18 g of $NaHCO_3$. All of the ingredients were dissolved in distilled water until homogeneous, then the solution was filtered until it was free from particles or dirt (Julianti *et al.,* 2018).

Microalgae nutrition was prepared by weighing 33 g of $FeCl_3$ (ion source) with 35 g of EDTA (buffer), 45 g of Dextrose (source of energy), 84 g of $KNO_3$ (nitrogen source), 54 g of TSP (phosphate source) with 18.9 g of EDTA (buffer) then dissolved in 1 L of distilled water (Primaryadi *et al.,* 2015).

### *2.2 Microalgae cultivation*

The growth medium solution was made from 450 mL of ASW in a 1000 mL glass bottle as the reactor, and then sterilized by autoclave at 121°C for 15 mins. The ASW was kept at room temperature. The pH was checked and the salinity was 22-24 ppt. Nutrients and microalgae broodstock were added to the bottle containing ASW, the level of aeration and lighting at the inoculation were adjusted, and the growth was observed for 14 days (Julianti *et al.,* 2018). Cell density in microalgae during the cultivation process could be determined by a hemocytometer to calculate cell density using the following formula (Padang *et al.,* 2018).

$$N \times 10^4 \text{ cells/d}$$

Where N = the average number of cells in the square box and the total actual cell density in 1mL of media or water is $10^4$.

### *2.3 Wet biomass insulation*

After the cultivation process for 14 days, the aeration and lighting were turned off, and the microalgae were settled for one night. The separation process was performed by removing the growth media by centrifugating the resulting residue at a speed of 4000 rpm for 5 mins. Afterwards, the pellets were stored at 4°C.

### *2.4 Phycobiliprotein extract*

The freeze-thawing method was used for the extraction process. A total of 1 g of wet microalgae biomass was added to 15 mL of aqua pro injection solvent, and the mixture was vortexed for 20 s for three times. The pellets were frozen for 24 hrs at -4°C, then thawed at 25°C in a dark room until melted. Then, the supernatant phase was centrifugated at 3500 rpm for 15

https://doi.org/10.26656/fr.2017.8(6).524 

mins and purified using a cellulose membrane dialysis tube with weight molecular cut-off 12-14 kDa for 12 hrs at 4°C. The supernatant was an extract of phycobiliprotein pigments, and then the pigment extracts were dried using the freeze-drying method (Hidhayati *et al.,* 2020).

*2.5 Phycobiliprotein qualitative test*

Phycobiliprotein qualitative testing used a 0.1% ninhydrin reagent. Ninhydrin reagent was made of 0.1 g of ninhydrin powder and 100 mL of 95% ethanol. The sample was sufficiently dissolved in 1 mL of aqua bidest, put in a test tube and 5 mL of the ninhydrin reagent was added to it. The solution mixture containing water in a beaker was heated at 100°C for 15 mins. The test shows positive results marked with a change in the solution to a blue-purple colour (Laksmiwati *et al.,* 2019).

*2.6 Fourier transform infrared spectrophotometer*

The phycobiliprotein isolated from the microalgae *Chlorella pyrinoidosa* was characterized by functional groups using an FTIR instrument at a wave number of 4000-600 $cm^{-1}$. For measuring the wave numbers, the CPU and the monitor were turned on. Then the monitor and the FTIR tool were turned on as well. For running and processing data, the micro lab pc application and the micro lab lite application were used. The FTIR crystal, the iron press, and the spatula were cleaned. Then, the sample was entered until it covered the crystal. The results could be seen in the wave number in the spectrograph application.

*2.7 ABTS free radical-scavenging activity assay*

The ABTS assay was based on the method described by Vifta *et al.* (2019) using ascorbic acid as the standard. ABTS and samples were diluted with distilled water. A thousand ppm of each sample diluted in distilled water at various concentrations (30, 60, 90, 120, and 150 ppm) was added to 2:1 of the ABTS solution. Then the reaction mixture was incubated at room temperature for 30 mins, and the absorbance was recorded at the corresponding maximum absorption wavelength (734 nm).

The ABTS free radical-scavenging activity was expressed by inhibition (I%), calculated as follows: I% = (A blank – A sample)/A blank × 100; where A sample was the absorbance of ABTS solution mixed with the sample and A blank was the absorbance of ABTS solution mixed with a solvent.

*2.8 Scanning electron microscopy*

The morphology and particle size were viewed using SEM, and the cellular structure of the phycobiliprotein was observed using scanning electron microscopy. Under the best extraction conditions, the sample was dispersed in distilled water, followed by pipetting on a glass slide and drying at 45°C. Photographs were taken at 15 kV at the desired magnification (Rodrigues *et al.,* 2015) ImageJ and origin software were used to determine the particle size distribution from the digital image.

## 3. Results and discussion

*3.1 Cultivation*

Microalgae cultivation or culturation is a technique for cultivating microalgae in a controlled environment (Pratiwi *et al.,* 2019). Cultivation was carried out to increase the number of cells; therefore, the desired biomass can be obtained. Microalgae growth in the cultivation process is influenced by nutritional sources and environmental factors, such as light, pH, salinity, temperature and aeration (Noerdjito, 2019). In this study, the cultivation medium used was artificial seawater consisting of NaCl, KCl, $CaCl_2$, $MgSO_4$, $MgCl_2$ and $NaHCO_3$, with additional nutrients such as $FeCl_3$, EDTA, dextrose, $KNO_3$ and TSP. Artificial seawater is used as a cultivation medium because of the microalgae. *Chlorella pyrinoidosa* is a marine microalga (Hadiyanto and Azim, 2012). In cultivation, the materials used for growth media and nutrition perform several functions. The function of NaCl in artificial seawater is to maintain water salinity. KCL, which consists of K elements fulfils a function in the carbohydrate metabolism, and Cl elements in the photosynthesis process, precisely in chloroplast activity. Ca in $CaCl_2$ carries out a function in the process of cell division and regulates the distribution of photosynthetic results. Mg in $MgSO_4$ and $MgCl_2$ forms chlorophyll in the photosynthesis process and acts as a regulator of the absorption of P (Phosphorus) and K (Potassium) (Primaryadi *et al.,* 2015). $NaHCO_3$ as a carbon source plays an important role in the process of photosynthesis and the process of cellular respiration (Kristiawan *et al.,* 2018).

Nutrients in cultivation act as feed which aims to accelerate microalgae growth. The nutrients are divided into two types: macro-nutrients and micro-nutrients. Dextrose, $KNO_3$ and TSP are included in the macro-nutrients and $FeCl_3$ in the micro-nutrients (Primaryadi *et al.,* 2015). Dextrose is a polysaccharide carbohydrate that can be used as a carbon or energy source (Toy and Puspita, 2019). Carbon is an essential nutrient for helping microalgae grow by forming cell structures (Nurdin, 2020). $KNO_3$ is a nitrogen source that increases cell density; therefore, a high enough biomass can be obtained. Low nitrogen in cultivation can cause a stressful environment for cells that have an impact on

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decreasing cell division.

TSP is an inorganic fertilizer that acts as a source of phosphorus. Phosphorus has an essential role in cell metabolism as a form of structural and functional components needed by cells for the growth and development of microalgae (Arfah *et al.,* 2019). Nitrogen and phosphorus are essential in preparing protein compounds in cells; a deficiency of these two elements can cause a decrease in protein content in microalgae cells. The decrease in protein content is generally followed by the degradation of various cell components related to protein synthesis, such as chlorophyll and pigments (Primaryadi *et al.,* 2015). $FeCl_3$ is a source of ions that plays a role in microalgae growth. Fe is part of a protein that plays a role in carrying electrons in the light phase of photosynthesis and respiration. Fe that plants can absorb are $Fe^{2+}$ and $Fe^{3+}$. Fe plays a role in forming chlorophyll, carbohydrates, fats and proteins (Primaryadi *et al.,* 2015).

*Chlorella pyrinoidosa* was cultivated in 450 mL of artificial seawater and 24 mL of nutrients, with 450 mL of microalgae culture. Cultivation was carried out at 23 ppt salinity and pH 7 for 14 days. Salinity and pH can affect microalgae growth during the cultivation process; therefore, it needs to be adjusted to the type of microalgae cultivated. *Chlorella pyrinoidosa* can grow well at 0-35 ppt salinity and at pH 7-9 (Aprilliyanti *et al.,* 2016).

Salinity is a water quality parameter that can affect the osmotic pressure between the protoplasm of organic cells and the environment. High levels of salinity can cause the external ion concentration to increase and water to be released from the cell. Salinity that is too low can cause water to enter the cell and reduce the concentration of ions, resulting in cell damage (Setiasih *et al.,* 2020). An increase in salinity level can be caused by water evaporation, which can reduce the volume of water, causing an increase in the concentration of salts dissolved in it (Rukminasari *et al.,* 2014).

The degree of acidity (pH) is an environmental factor that must also be considered. pH can be described as the presence of hydrogen ions that affect the solubility and availability of mineral ions, thus affecting the absorption of nutrients by cells. During the cultivation process, changes in pH can occur; this is caused by the photosynthesis process of microalgae that use $CO_2$ in the form of carbonate ions ($CO_3^{2-}$) and bicarbonate ions ($HCO^{3-}$). The photosynthesis process can produce glucose and alkaline $OH^-$ ions, thus the pH during the cultivation process can increase. Changes in pH can cause inhibition of enzymes and the photosynthetic process of microalgae. An acidic pH can interfere with cell metabolic processes. It causes a decrease in the ability to absorb nutrients, thereby affecting cell growth. At the same time, a pH that is too alkaline can cause a reduction in the work of enzymes to form ammonium which is used in microalgae metabolism and consequently, it can reduce the density of microalgae cells (Richmond, 2018).

Artificial seawater used in cultivation must be clear and free from impurities; therefore, it must be filtered first before use, otherwise, it can inhibit the photosynthesis of microalgae because impurities block the light (Supriyantini, 2013). In the process of making nutrients, $FeCl_3$ and TSP were dissolved in $Na_2EDTA$ because $FeCl_3$ was only soluble in acidic conditions. In addition, it aims to maintain and stabilize Fe and TSP under varying pH conditions (Armanda, 2013).

*Chlorella pyrinoidosa* is an autotrophic organism capable of forming food or organic compounds derived from inorganic compounds through photosynthesis (Richmond, 2018). One of the characteristics of microalgae is photoautotroph, which requires light as an energy source for cell growth and to synthesize essential substances in microalgae. The light source used in the cultivation process was a 19-watt lamp (Philips). Microalgae lighting was carried out based on its natural day-night rhythm in which it was given light for 12 hrs during the day and 12 hrs during the night. Low light intensity can inhibit the photosynthesis process. The optimal light intensity for microalgae growth is 1500-3000 lux and not more than 4000 lux to avoid photoinhibition. Photoinhibition is excess light intensity captured by chlorophyll, causing a decrease in the photosynthetic ability of chlorophyll (Richmond, 2018).

The aeration cultivation process continued for 14 days except during sampling for the calculation of cell density. Aeration aims to maintain the temperature in the bottle to remain the same and evenly distribute nutrients. Circulation of water resulting from the aeration process can also prevent the deposition of microalgae. The aeration process can produce water bubbles which help increase $O_2$ levels from the air into the water, thereby increasing the contact of water with oxygen in the air (Fitriani *et al.,* 2017). The growth phase of *Chlorella pyrinoidosa* during cultivation was described by daily cell density calculations using a hemacytometer. Cell density calculations were carried out to know the growth phase of microalgae (Richmond, 2018). Microalgae cell density can change because it is influenced by internal factors, such as microalgae type, cell shape, size, and cell physiology and external factors, such as pH, light intensity and salinity (Zainuddin *et al.,* 2017). Figure 1 shows a curve of the growth rate of *Chlorella pyrinoidosa* for 14 days.

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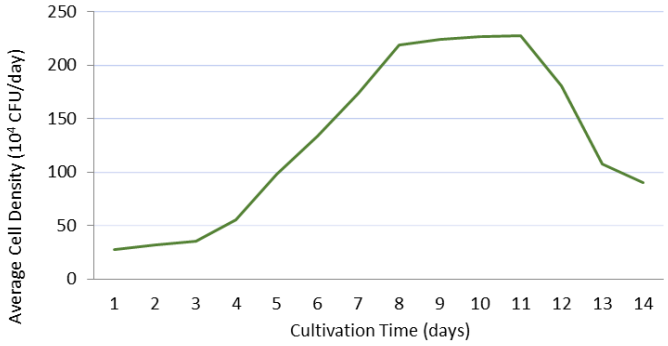


Figure 1. *Chlorella pyrinoidosa* growth rate curve.

Based on the growth rate curve of *Chlorella pyrinoidosa*, Table 1 shows that the cell density of *Chlorella pyrinoidosa* follows the general microalgae growth phase pattern, which consists of an adaptation phase, an early growth phase, an exponential phase, a slowed growth phase, a stationary phase, a dying phase and a death phase (Boleng, 2015). Day 1 to day 4 is the adaptation phase where the microalgae are still trying to adapt to the new environment. This phase is characterized by the low value of cell density and the slow process of cell division (Prayitno, 2016). Next is the initial growth phase which occurs on day five and is marked by increasing cell density. However, the speed of cell division in this phase is still relatively low (Boleng, 2015). After experiencing the initial growth phase, microalgae enter the exponential or logarithmic phase on days 6 to 8. A rapid increase in cell density characterizes this phase. Microalgae use more nutrients in this phase than in other phases. Using many nutrients leads to a decrease in nutrients in the growth media which causes the microalgae to enter a slowed growth phase. This phase occurs because microalgae lack nutrients, causing a reduction in growth rate (Boleng, 2015). The delayed growth phase occurs adjacent to the stationary phase.

Table 1. *Chlorella pyrinoidosa* growth rate.

| Cultivation time (days) | Average cell density ($\times 10^4$ CFU/d) | Description |
|---|---|---|
| 1 | 27.25 | adaptation phase |
| 2 | 31.87 | |
| 3 | 35.25 | |
| 4 | 55.44 | |
| 5 | 97.75 | initial growth phase |
| 6 | 133.50 | slow growth phase |
| 7 | 173.31 | |
| 8 | 219.00 | |
| 9 | 224.25 | stationary phase |
| 10 | 226.56 | |
| 11 | 227.37 | |
| 12 | 180.50 | dying phase |
| 13 | 107.50 | death phase |
| 14 | 89.87 | |

The stationary phase occurs on days 9-11 in which the density of the microalgae cells is relatively constant because the cell density is already quite dense, and nutrients are depleted (Prayitno, 2016). On day 12, microalgae began to enter the dying phase because the growth media formed toxic substances that cause cell death, decreasing cell density (Boleng, 2015). After the dying phase, microalgae entered the death phase on days 13-14 which saw a relatively rapid decrease in cell density due to a decline in growth factors such as nutrients and oxygen. This causes microalgae to no longer be able to perform cell division (Yanuaris *et al.,* 2012).

During the 14 days of the cultivation process, the microalgae growth media changed colour and eventually became greener. This indicates that microalgae are multiplying, and as a result, many microalgae deposits are formed and change the colour of the culture each day.

### *3.2 Extraction*

Extraction is a process to obtain the compound of interest; therefore, it is necessary to suit the method in this process (Hidhayati *et al.,* 2020). Extraction of *Chlorella pyrinoidosa* begins with a wet biomass isolation process to separate the wet biomass from the growth media solution. The separation was carried out after an overnight deposition process from the completion of the cultivation process using centrifugation (Salim *et al.,* 2018). Centrifugation is a tool used to separate solid and liquid mixtures. The working principle of this tool is applying centrifugal force by rotating to perform separations based on specific gravity (Hidayah *et al.,* 2014). Wet biomass isolation was centrifuged at a speed of 4000 rpm for 5 mins. The biomass of *Chlorella pyrinoidosa* was light green. The pellets obtained were weighed, and the yield was calculated and then stored in a cooler at 4°C. The higher the cell density value, the higher the wet biomass weight will be (Hidayah *et al.,* 2014).

After the separation process, the pellets were extracted using the freeze-thawing method, which is the most efficient extraction method for phycobiliproteins extracted from wet biomass (Hidhayati *et al.,* 2020). Freezing was carried out for 24 hrs in the freezer, and thawing was carried out until the solution melted at room temperature. The freezing process results in the formation of intracellular ice that causes cell wall rupture (Vernès *et al.,* 2015), and the thawing process can cause cellular contraction (Neti *et al.,* 2018). The primary purpose of this method is to stress cells to accelerate the release of pigment (Rahmawati *et al.,* 2017).

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The choice of solvent is essential in the extraction process. The solvent must be able to dissolve the target compound to yield a maximum amount of extract (Rahmawati *et al.,* 2017). As phycobiliproteins are polar pigments, the solvent must be polar. Although phosphate buffer was often used, based on several studies, the best solvent for phycobiliprotein extraction was distilled water (Tan *et al.,* 2020). Compared to phosphate buffers, water attracts phycobiliproteins better, is more economical, and is non-toxic (Karseno *et al.,* 2013). Besides the solvent, pH is also important to consider; distilled water with a pH of 7 is the best solvent for phycobiliprotein extraction. pH can affect the charge of phycobiliproteins by increasing the solubility in the charged state and inhibiting the solubility in the neutral state. The isoelectric point of phycobiliprotein is 4.74 - 5.8. Solvents with a pH higher than the isoelectric point can increase solubility (Tan *et al*., 2020). After the extraction process finished, the melted solution was centrifuged at a speed of 3,500 rpm for 15 mins to separate the pellet from the filtrate. The solution that was centrifuged was purified using a cellulose membrane dialysis tube weight cutoff of 12 -14 kDa at 4°C for 12 hrs to remove salts, minerals and carbohydrates. The filtrate was then dried using the freeze-drying technique to maintain the stability of phycobiliproteins because phycobiliproteins are easily oxidized when exposed to high heat and light intensity and to maintain the colour of the phycobiliproteins (Agustini, 2012). The phycobiliprotein extract obtained was 0.0724±0.0012 g/ mL.

### *3.3 Phycobiliprotein qualitative test*

A qualitative test of phycobiliprotein was carried out using an amino acid test with the ninhydrin test method to detect the presence of free amino acids in the substance tested (Lestari *et al.,* 2019). Ninhydrin acts as an oxidizing agent that causes oxidative decarboxylation of amino acids, which can produce hydridantine, $CO_2$, $NH_3$, and aldehydes with a C chain shorter than the original amino acid. The reaction of reduced ninhydrin with $NH_3$ can form a blue-purple-colored complex. The more ninhydrin in the test substance, the more concentrated the color is (Laksmiwati *et al.,* 2019). Based on the tests carried out on the phycobiliprotein extract powder, it was found that the positive sample contained protein. This was indicated by a change in the color of the sample solution from light green to purple (Laksmiwati *et al*., 2019).

### *3.4 Fourier transform infrared spectrophotometer*

FTIR spectroscopy is a test method to obtain information about the types of vibrations between atoms in minerals and provides information about functional groups in the structure of compounds (Hardjono, 2013).

The analysis is usually carried out in the wave number area of 400-4000 $cm^{-1}$ because spectra with characteristics will appear in the wave area (Hardjono, 2013). Figure 2 shows the FTIR results from the phycobiliprotein extract showing four absorption bands (peaks), where the absorption band in the wave number region which was less than 1400 $cm^{-1}$ was the fingerprint region. In the wavenumber region, the functional groups range from 1400-4000 $cm^{-1}$(Veronika *et al.,* 2017).

Table 2 shows the identification result of the phycobiliprotein spectrum. The broad absorption at wave number 3257 $cm^{-1}$ indicates the presence of a hydroxyl group (OH), which is a type of carboxylic acid vibration. The OH group was found at the peak of the vibration wave number 2400-3400 $cm^{-1}$. This widening of the absorption band of the hydroxyl group causes the absorption band of the N-H group to be closed, appearing in the 3100-3500 $cm^{-1}$ region (Rodrigues *et al.,* 2015). However, the N-H group can still be recognized by the appearance of an absorption band at a wave number of 1635 $cm^{-1}$, which indicates the presence of a carbonyl group (C=O) with the type of amide vibration (Veronika *et al.,* 2017). The presence of N-H

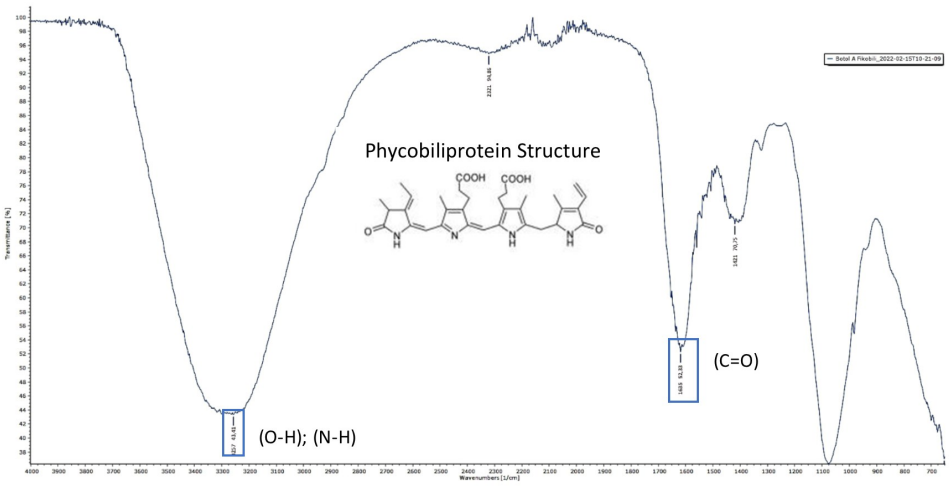


Figure 2. *Chlorella pyrinoidosa* FTIR.

https://doi.org/10.26656/fr.2017.8(6).524 

and C=O groups in this phycobiliprotein extract can make the extract easily soluble in water because it allows the formation of hydrogen bonds. The results of this test have specific functional groups of phycobiliprotein extracts, which are the presence of functional groups O-H, N-H, and C=O. These results from the spectrum of phycobiliproteins in the microalgae *Spirulina arthrospores* (Rodrigues *et al.,* 2015) and one of the phycobiliprotein derivatives, phycoerythrin, derived from the seaweed *Eucheuma cottoni* (Veronika *et al.,* 2017).

Table 2. Identification result of Phycobiliprotein spectrum.

| No | Wave number | Group | Frequency | Vibration Type |
|---|---|---|---|---|
| 1 | 3257 ($cm^{-1}$) | O-H | 2400-3500 ($cm^{-1}$) | Carboxylic Acid |
| 2 | 3257 ($cm^{-1}$) | N-H | 3100-3500 ($cm^{-1}$) | Amida |
| 3 | 1635 ($cm^{-1}$) | C=O | 1630-1680 ($cm^{-1}$) | Amida |

### *3.5 Scanning electron microscopy*

The results of the phycobiliprotein morphology test from *Chlorella pyrinoidosa* showed that the phycobiliprotein had a crystal-like shape, most of which were almost the same size. Based on research by Rodrigues *et al.* (2015), the morphology of the microstructure size of phycobiliproteins is in the form of small granules that are unevenly distributed on the surface and have cracks and quite diverse clusters. The results of this study are based on the results of the SEM test.

The digital SEM images of the phycobiliproteins (Figure 3) were then processed using image-J and Origin applications to analyze the distribution of the particle sizes. Particle measurements were carried out using the J -image, while the distribution of the measurement results using the Origin (Sasri *et al.,* 2018). Based on the results of the particle size calculations, the average diameter of phycobiliproteins was 10 μm. After measuring the particles, the next phase was the distribution of the results. In the particle size distribution, the value used was the diameter or length value of the overall data. The diameter from these measurements is then made in the form of a plot. Based on the plot, the highest particle size frequency was in the range of 8,000 to 10,000 nm, and the lowest was in the field of 18,000 to 20,000 nm.

### *3.6 ABTS free radical-scavenging activity assay*

Phycobiliprotein antiradical was tested using the ABTS method. The ABTS method is based on the ability of a compound to stabilize free radicals by donating proton radicals (Imrawati *et al.,* 2017). The donation of protons to radical compounds can inhibit the reactions from forming free radicals by completing the electron deficiency in the free radicals; therefore, the free radicals become more stable compounds (Setiawan *et al.,* 2018). This method was chosen because the ABTS method is more sensitive, can be used in various pHs, and is soluble in water and organic solvents. The reaction occurs faster and can be used for many compounds (Salampe *et al.,* 2019). However, the ABTS method is susceptible to light; even forming ABTS radicals requires 12-16 hrs of incubation in dark conditions (Vieira *et al.,* 2017).

The principle of testing antiradical activity using the ABTS method is by changing the intensity of the blue-green color on ABTS free radicals that do not have an electron pair and will turn clear or fade when the electrons are paired (Salampe *et al*., 2019). The change in color intensity also indicates the process of reducing free radicals produced by the reaction between the ABTS molecules and the protons released by the sample compound molecules. This color change results in a difference in the absorbance value at the maximum wavelength of ABTS using a UV-Vis spectrophotometer; therefore, the value of the free radical scavenging activity of phycobiliproteins can be determined, which was expressed by the inhibitory concentration ($IC_{50}$) value (Imrawati *et al.,* 2017). The $IC_{50}$ value is defined as the concentration of a test compound that can reduce 50% of free radicals. The smaller the $IC_{50}$ value, the higher the free radical scavenging activity may become, and the smaller the concentration, the greater the absorbance will be (Salim, 2018). $IC_{50}$ values can be grouped into activity ranges, including <50 ppm (very

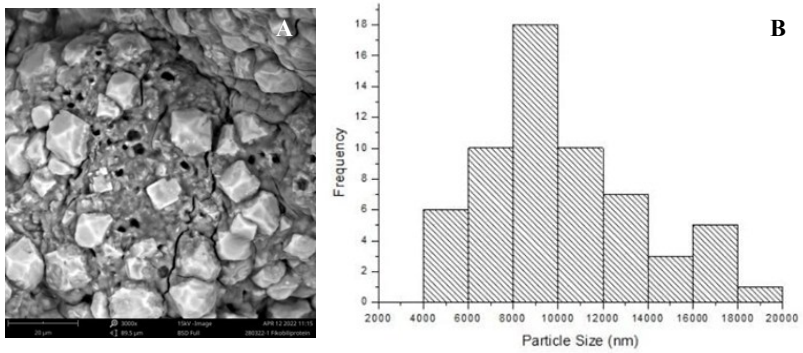


Figure 3. Phycobiliprotein (a) morphology 3000× magnification, (b) particle size plot.

https://doi.org/10.26656/fr.2017.8(6).524 

strong activity), 50-100 ppm (strong activity), 100-250 ppm (moderate activity), and 250-500 ppm (weak activity) (Rizki *et al.,* 2021).

Quantitative testing of phycobiliprotein antiradical activity was carried out with various concentration series using the ABTS method, whose absorbance was measured by a UV-Vis spectrophotometer. Measurement of phycobiliprotein absorbance begins by determining the maximum wavelength of ABTS. The maximum wavelength of ABTS used is in the range of 400-800 nm (De Oliveira *et al.,* 2014)). The maximum wavelength of ABTS is the wavelength that can provide the maximum absorption of the test solution and provides the highest sensitivity. Based on the measurement results, the maximum wavelength of ABTS was at a wavelength of 734 nm. The antiradical activity of the phycobiliproteins and the comparison used were measured at the maximum wavelength (Amin *et al.,* 2021).

The comparison used in this study was vitamin C. Vitamin C is a natural antiradical and is often used as a comparison because it has higher antiradical activity when compared to vitamins A and E, is easy to obtain, inexpensive, relatively safe, and does not cause toxicity. Vitamin C has efficacy as an antiradical because vitamin C has free hydroxy groups that act as free radical scavengers (Purwanti *et al.,* 2019). Based on the measurement results, the absorbance of ABTS was 0.843.

A quantitative antioxidant activity test was carried out using a UV-Vis spectrophotometer to determine the remaining ABTS absorbance after adding phycobiliprotein extract. The decrease in the absorbance value at a maximum wavelength of 734 nm at higher concentrations and the color change from blue-green to fading indicates the presence of antioxidant activity in the phycobiliprotein extract. The absorbance value can be used to calculate the percentage of free radical inhibitors (% inhibition) (Rizki *et al.,* 2021). Meanwhile, to calculate the $IC_{50}$ value, a linear equation from a linear regression curve can be used, which consists of concentration on the x-axis and absorbance on the y-axis. Percentage inhibition can be calculated using a linear equation from the curve between % inhibition and concentration. Based on the % inhibition curve with concentration, the linear equation value for vitamin C is $y = 11.333x + 10.929$ with $R^2 = 0.9967$ and for phycobiliprotein is $y = 0.3187x + 4.9822$ with $R^2 = 0.9988$. When calculating the % inhibition variable, y was replaced by 50 (Amin *et al*., 2021). Based on the $IC_{50}$ calculation results, vitamin C was a strong antioxidant with an $IC_{50}$ value of 3.447 ppm. In comparison, phycobiliprotein is a moderate antioxidant with an $IC_{50}$ value of 141.254 ppm. The difference in these values can be caused by the ability of these compounds to donate electrons to ABTS radicals, where the more electrons are given, the more the absorbance value will decrease, and as the % inhibition increases, the $IC_{50}$ value will decrease (Vifta *et al.,* 2019).

Phycobiliproteins were extracted using the freeze-thawing method and tested for antioxidant activity using the DPPH method from the microalgae *Spirulina platensis* (Rahmawati *et al.,* 2017) Arthospira sp. (Rodrigues *et al.,* 2015), and *Chroococcus turgidus* (Hidhayati *et al*., 2020) has an $IC_{50}$ value in the very strong to moderate range. The DPPH method produces a different $IC_{50}$ value from the ABTS method, even though it is carried out on the same sample and extraction method. This is because the ABTS method is more sensitive and able to provide better antioxidant indicators than the DPPH method; therefore, the $IC_{50}$ results in samples using the ABTS method can be higher (Salampe *et al*., 2019). In addition, the high and low antioxidant activities of a sample can be influenced by the type of solvent used (Happy *et al.,* 2019).

## 4. Conclusion

Based on the results of the research, *Chlorella pyrinoidosa* has phycobiliprotein compounds with the characteristics of having O-H groups. These compounds also possess N-H and C=O, and have the shape of small granules that are unevenly distributed on the surface. There are cracks and clusters that are quite diverse, with an average diameter of 10 μm. Phycobiliprotein in *Chlorella pyrinoidosa* has moderate antiradical activity with an $IC_{50}$ value of 141.254 ppm using the ABTS method. Therefore, the phycobiliproteins compound in *Chlorella pyrinoidosa* has the potential to be used as a food supplement.

## Conflict of interest

The authors declare no conflict of interest

## Acknowledgements

The authors would like to thank the Institute for Research and Community Service of Bakti Tunas Husada University.

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