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Research Article

Biochemical Processes of *Chlorella vulgaris* and Their Impact on Chlorophyll Quality and Antioxidant Properties

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Abstract

Chlorella is a microalga that is rich in chlorophyll and antioxidants so it has the potential to be a functional food or health supplement; however, the quality of *Chlorella* depends on the nutrient composition in cultivation. The research aimed to evaluate the effect of different formulations in *Chlorella* cultivation on the content of chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and antioxidants. Furthermore, to analyse the profile of amino acids, fatty acids and secondary metabolism in the best formulation. The experimental design used was a non-factorial Completely Randomized Design (CRD) with 5 formulations in *Chlorella* cultivation: F-1, F-2, F-3, F-4, and F-5 by manipulating the use of 4 chemicals: KNO_3, KH_2PO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$. The data obtained were analyzed descriptively and analysis of variance (ANOVA). The results showed that F-2 treatment with the use of 1.50 KNO_3, 1.25 KH_2PO_4, 1 $MgSO_4.7H_2O$ and 0.0498 $FeSO_4.7H_2O$ (g/L) was the best treatment with the content of chlorophyll a 38.19 µg/mL, chlorophyll b 41.45 µg/mL, total chlorophyll 79.65 µg/mL, carotenoids 0.08 µg/mL, and antioxidants activity 49.52 mg/L (strong) which is the same as the F-1 treatment. In addition, *Chlorella* cultivated with the F-2 formula has 17 amino acid profiles with a total of 301.52 mg/g, 7 fatty acids 84.32 mg/g, and secondary metabolites, namely alkaloid 109.471 mg/L, flavonoid 82.111 mg/L, saponin 1342.222 mg/L, tannin 411,591 mg/L, and phenolic 151.889 mg/L. Therefore, the F-2 formulation can be developed for large-scale *Chlorella* cultivation and applied as a health supplement.

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1. Introduction

The estuary of the Rokan River, located in Bangko Subdistrict, Rokan Hilir District, Riau, Indonesia, is a nutrient-rich environment, largely due to its proximity to the Strait of Malacca. This region is ideal for studying *Chlorella vulgaris*, a green microalga known for its chlorophyll content and antioxidant properties. Given the environmental conditions, *Chlorella* cultivation could offer valuable insights into improving its nutritional and health benefits. One of the phytoplankton found in these waters is *Chlorella vulgaris* This *Chlorella* has been isolated and purified, and it has a size of 8-10 µm (Iriani *et al*., 2021). *Chlorella* is one of the green microalgae that is rich in chlorophyll (Amelia *et al*., 2023; Yuniarti *et al*., 2023). Chlorophyll can absorb metals to give a green pigment, which causes the structure of chlorophyll to consist of a tetrapyrrole ring called porphyrin bonded to a magnesium (Mg^{2+}) atom in the middle.

Chlorophyll in *Chlorella vulgaris* plays a key role in neutralizing free radicals (ROS) that damage macromolecules such as lipids, proteins, and DNA, contributing to conditions like cancer, diabetes, aging, and neurodegeneration (Ngo *et al.,* 2011; Roy *et al.,* 2023). The antioxidant capacity of chlorophyll is closely tied to biochemical processes such as the synthesis of sugars, amino acids, fatty acids, and secondary metabolites, all of which enhance its ability to combat oxidative stress. However, the quality of chlorophyll also depends on biochemical processes that are closely related to the formation of sugars, amino acids, fatty acids and secondary metabolism (Wang *et al*., 2021) to increase antioxidant properties in reducing free radicals. Better photosynthesis in forming chlorophyll can improve nutritional and antioxidant quality (Brown *et al*., 1993; Barkia *et al*., 2019).

The nutrient content of *Chlorella vulgaris* is greatly influenced by macronutrients such as nitrogen (N), phosphorus (P), and potassium (K), as well as micronutrients like iron (Fe) and magnesium (Mg). These nutrients are essential for photosynthesis, chlorophyll production, and overall metabolic functions. For example, the KH_2PO_4 compound plays a vital role in enzyme activation, stomatal opening, and the regulation of plant growth hormones (Chewapanich *et al.,* 2021). The elements Nitrogen (N), Phosphorus (P), and Potassium (K) are the main elements in cultivation (Thongpitak *et al*., 2018; Yu *et al*., 2021; Elbasuney *et al*., 2022; Wang *et al*., 2023).The function of KH_2PO_4 compound is enzyme activation, stomatal opening, embryo-somatic development and cytokine hormones (Maisarah *et al.,* 2020; Chewapanich *et al*., 2021). Another role of Fe and Mg is as formers and catalysts in chlorophyll synthesis (Farhat *et al*., 2016; Elbasuney *et al*., 2022). Furthermore, NO_3^- and NH_4^+ are more common sources of nitrogen than KNO_3 for microalgae (Raven and Giordano, 2016). The use of ammonium-N (NH_4NO_3) as a nitrogen source in cultivation can increase the growth rate and lipid content of microalgae species Tetraselmiss, Spirulina, *Scendesmus* sp and *Chlorella vulgaris* (Kim *et al*., 2016; Li *et al*., 2019; Salbitani and Carfagna, 2021; Carletti *et al*., 2024), while the use of nitrate-N is better for cell growth Tetraselmis, because it produces twice as much biomass as ammonium (NH_4NO_3) (Kim *et al*., 2016).

Most of the media for cultivating microalgae contains many chemicals such as Basal media that is used for cultivating *Chlorella* sp in Thailand with 12 chemicals: KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, EDTA, H_3BO_3, $CaCl_2.2H_2OH$, $ZnSO_4.7H_2OH$, $FeSO_4.7H_2OH$, $CuSO_4.5H_2O$, $MnCl_2.4H_2OH$, MoO_3, and $Co(NO_3)_2.6H_2O$) (Iriani *et al*., 2011), cultivating *Chlorella* for face mask (Iriani *et al*., 2023). Furthermore, in cultivating green microalgae *Pediastrum duplex* using a Jaworski medium containing 14 chemicals, Andersen (2005) and Thongpitak *et al*. (2018) found that a medium using four chemicals containing $NaNO_3$, KH_2PO_4, $NaHCO_3$, and $MnSO_4$ was able to support microalgae growth similarly to the Jaworski medium. It was manipulated by the use of Bold's Basal Medium consisting of 13 ingredients into five chemicals for *Chlorella* cultivation using the N/P ratio (Wong *et al*., 2017), and the addition of wastewater desalination concentrate as an alternative media (Matos *et al*., 2018). Furthermore, various *Chlorella* culture media, NPK+urea manipulation media are the best growth media compared to F/2, Walne, Jarowski (Indrayani *et al*., 2023), and Bold's Basal Media enriched with sodium acetate and urea, phosphate, and potassium can increase biomass and nutritional content of *Chlorella* (Khan *et al*., 2020). In addition, TAP, BG-11, AF6, and F-2 media are enriched with sodium hydroxide to increase *Chlorella* biomass (Sadewo *et al*., 2022), and BG-11 media are enriched with different doses of glucose, sodium nitrate, and dipotassium phosphate as a substitute for organic carbon sources in increasing *Chlorella* biomass (Kim *et al*., 2019; Yun *et al*., 2021). Walne media, with the addition of different doses of Fe^{3+} can also produce *Chlorella* to maximize economic benefits (Rakhmadumila and Muntalif , 2020; Cheng *et al*., 2022). Furthermore, Ajala and Alexander (2020) have researched the use of nitrate and phosphate to remove nitrate sulfate and phosphate in wastewater. Cheap alternative media for large-scale production can be obtained by manipulating the media using wheat bran fermentation concentration (Akter *et*

al., 2022) and tofu water waste (Arsad *et al*., 2020).

The *Chlorella* used in this research was *Chlorella* that had just been isolated from the waters of the Rokan River Estuary, where it had previously been cultivated using synthetic media, but its growth was not optimal (Iriani *et al*., 2021; Amelia *et al*., 2023; Erfianti *et al*., 2023; Yuniarti *et al*., 2023). For large-scale biomass production, an optimal and efficient nutrient media composition is required for efficient production costs. Considering that there are too many chemicals used in commercial cultivation media (Andersen, 2005; Iriani *et al*., 2011), these materials are difficult to obtain and are not economical. For this reason, in this research, four types of chemical compounds, namely KNO_3, KH_2PO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ were manipulated to obtain the optimal medium formulation for the biochemical compounds of *Chlorella vulgaris,* which was isolated from the waters of the Rokan River estuary, Riau, Indonesia. Due to no information being available about the nutrient formulation of the medium for biochemical compounds, *Chlorella* was isolated from these waters. However, all previous studies were focused on the growth of *Chlorella* and, to our knowledge, very few studies have been conducted concerning the media formulation which can increase the chlorophyll, antioxidant content, amino acids, fatty acids, and quantitative secondary metabolite of *Chlorella* isolated from Rokan River estuary. This biochemical compound is useful for determining the potential of *Chlorella* as a health supplement (Bito *et al*., 2020; Hanieh *et al*., 2023; Mavrommatis *et al*., 2023).

Studies about how using minimal chemicals in *Chlorella* cultivation (KNO_3, KH_2PO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$) can improve its biochemical compounds are still relatively limited. Hence, the research aimed to evaluate the effect of different formulations in *Chlorella* cultivation on the content of chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and antioxidants. Furthermore, should be analysed the profile of amino acids, fatty acids and secondary metabolism in the best formulation. The results of this study are expected to reduce chemical material and cost in the cultivation of *Chlorella*. The use of these four chemicals can replace the commercial media of *Chlorella* cultivation; however, it contains high biochemical compounds. So, it can be used as a health supplement.

2. Materials and Methods

2.1 Materials

The *Chlorella* was isolated from the Rokan River estuary, Riau province, Indonesia, with coordinate points at latitude 2.258832 (2°15'31.8"N), longitude 100.751340 (100°45'04.8"E), and width 346.60 m. (Figure 1). After pure *Chlorella* was obtained (Figure 2), it proceeded to mass culture using different formulations. *Chlorella* was cultivated for 16 days at a temperature of 25 ± 2°C, a humidity of 45%, with 24-hour lighting, and used sunlight during the day, while at night it used a 36 W TLD lamp (5000 lux meter). After the 16th day, harvesting was carried out using a centrifuge at 4000 rpm, temperature 11^{O}C, for four minutes, and then lyophilized with freeze drying to obtain the *Chlorella* powder.

2.1.1 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

2.2.1 Culture

The design used was a non-factorial Completely Randomized Design (CRD) with five treatment levels consisting of culture media formulation. In a F-1 treatment using commercial media with 12 chemicals: KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, EDTA, H_3BO_3, $CaCl_2.2H_2OH$, $ZnSO_4.7H_2OH$, $FeSO_4.7H_2OH$, $CuSO_4.5H_2O$, $MnCl_2.4H_2OH$, MoO_3, and $Co(NO_3)_2.6H_2O$), F-2, F-3, F-4, and F-5 are treatment media formulated by increasing the concentration of macronutrients and eliminating micronutrients in the F-1/commercial media, except $FeSO_4.7H_2O$. For more details, see Table 1.

Culture media (F-1, F-2, F-3, F-4, and F-5) were made by dissolving all the chemicals in each formulation into 1 L of sterile distilled water. A total of 100 ml of seeds *Chlorella* were cultured into 900 ml of pH 7 culture medium and grown in a controlled culture room. The culture was shaken every morning to avoid nutrient buildup.

2.2.2 Analysis of chlorophyll and carotenoid levels

Analysis of chlorophyll a, chlorophyll b and carotenoid levels *Chlorella* was according to (Lichtenthaler, 1987). The *Chlorella* was extracted with 96% methanol solvent (5 mg/5mL) and then measured using a spectrophotometer with wavelengths (Abs) 652, 665 and 470 nm. Then, levels of chlorophyll a, chlorophyll b and carotenoids were calculated using the formula below:

Chlorophyll a (µg/mL) = 16.72 (Abs 665) - 9.16 (Abs 652)

Chlorophyll b (µg/mL) = 34.09 (Abs 652) - 15.28 (Abs 665)

Total chlorophyll (µg/mL) = 1.44 (Abs 665) + 24.93 (Abs 652)

Carotenoids (µg/mL) = 1000 (Abs 470) – 1.63 (Ca)-104.96 (Cb)/221

Where :

Ca : Chlorophyll a content (µg/mL)

Cb : Chlorophyll b content (µg/mL)

Abs 652 : Absorbance value at a wavelength of 652 nm

Abs 665 : Absorbance value at a wavelength of 665 nm

Abs 470 : Absorbance value at a wavelength of 470 nm

methanol solution. The sample solution was diluted to obtain a sample solution with a concentration series, namely 1.000, 500, 250, 125, 6.25, 31.25 ppm. Each sample solution concentration was taken as 4 mL and reacted with 1 mL of DPPH solution, 0.2 M. The absorbance of the sample was measured using a spectrophotometer at a wavelength of 517 nm.

% inhibition= ×100%

Note, A = absorbance of DPPH solution, and B = absorbance of DPPH and extract solution

The sample concentration values and percent inhibition were plotted respectively on the x and y axes in the linear regression equation, the equation formed is y=a+bx, used to find the IC_{50} value (inhibitor concentration 50%) of each sample tested by stating the y value of 50 and the x value that will be obtained as

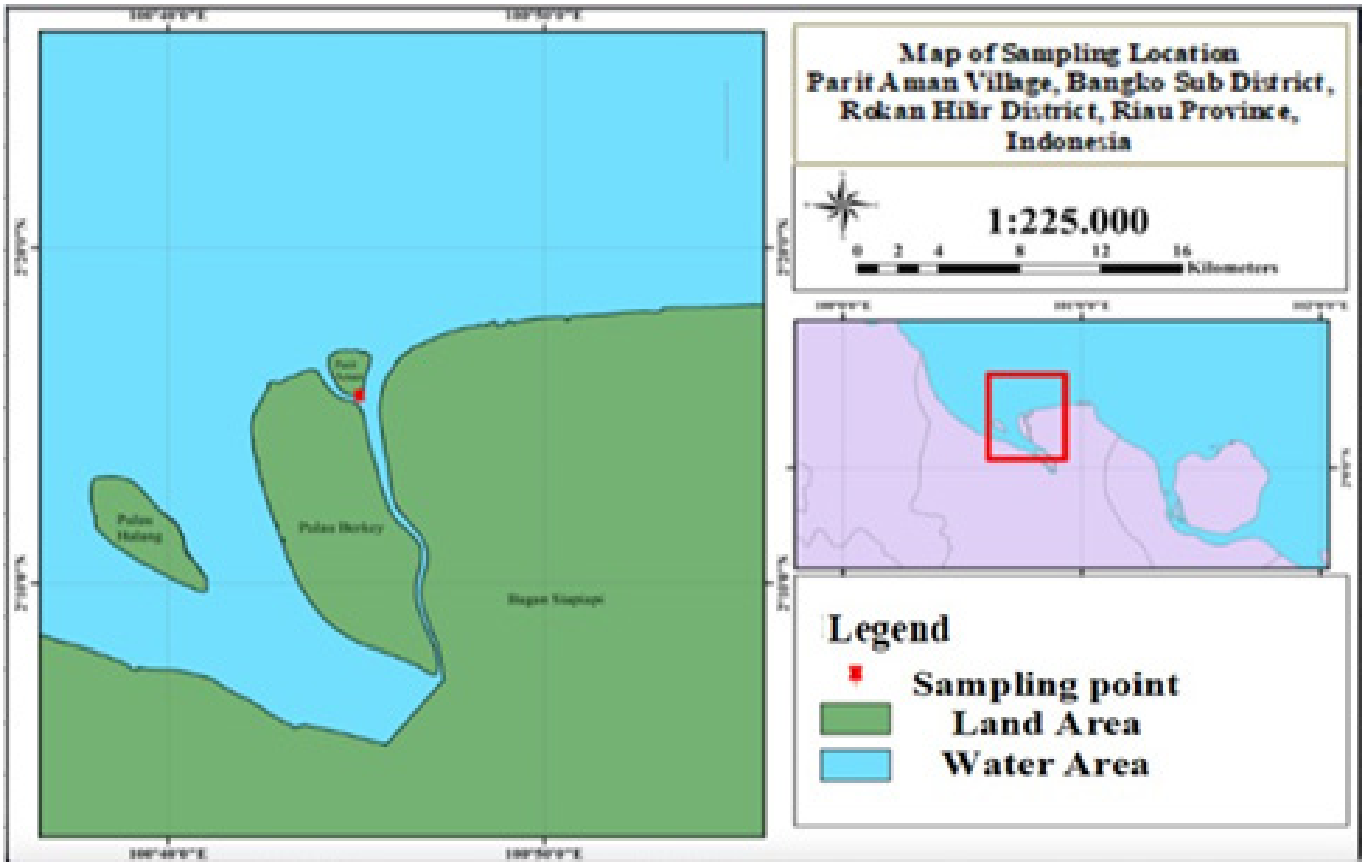

Figure 1. Sampling location of Chlorella vulgaris in the Rokan river estuary.

2.2.3 Analysis of antioxidant activity

Determination of antioxidant activity was carried out based on the DPPH method, as referred to by Molyneux (2004). *Chlorella* pasta was extracted with methanol. The solution was filtered and antioxidant analysis was carried out. The sample was made into a stock solution with a concentration of 1.000 ppm in

IC_{50}, then linear regression (scatter) was carried out on each treatment on the inhibition value (%) and inhibition concentration using Microsoft Excel 2016 to see the correlation in inhibiting free radicals.

2.2.4 Chlorella extraction

A total of 50 g of dry sample *Chlorella* was

macerated with a mixed solvent of methanol: acetone (7:3), at a rate of 1:10 (w/v) for three days, every 24 hours and filtered using vacuum filtration (modification of Rowan, 1989), then the sample was extracted with a rotary evaporator until condensed at a temperature of 40°C. After that, the sample was partitioned using a separating funnel using n-hexane solvent (1:5) and the bottom layer was taken then a rotary evaporator was used until it concentrated at a low temperature.

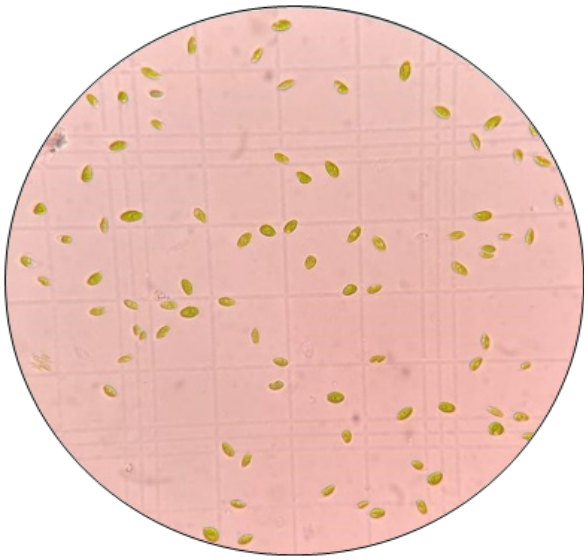

Figure 2. *Chlorella vulgaris.*

2.2.5 Amino acid analysis

The amino acid profile was analysed according to AOAC (2005). A total of 30 µL of extract *Chlorella* was mixed with a mixed solution (methanol: picothiocyanate: triethylamine) in a ratio of 4:4:3, and diluted by adding 20 mL of 60% 1M sodium acetate buffer, then left for 20 minutes. The dilution results were filtered again using a 45-micron millipore. Next, 40 µL of the filter results were taken to be injected into the HPLC (temperature 27°C and a wavelength of 254 nm). The concentration of amino acids in the material was calculated by making a standard chromatogram.

2.2.6 Fatty acid analysis

The fatty acid profile was analyzed according to AOAC (2005). As much as 5 g *Chlorella* was extracted with soxhlet to obtain 20-40 mg of fat. The extraction results were heated by adding 1 mL of 0.5 N NaOH and methanol for 20 minutes, then cooled. Then, 2 mL of 20% BF3, 2 mL of saturated NaCl and 1 mL of n-hexane were added and shaken until homogeneous. The n-hexane layer was transferred into a tube containing 0.1 g Na_2SO_4, for 15 minutes. The liquid phase was separated and injected at temperatures ranging from 125-240°C. The type and amount of fatty acids can be identified by comparing the peak chromatogram of materials with standards.

Table 1. Different formulations in the *Chlorella* cultivation.

	Treatments				
Chemicals (g/L)	**F-1**	**F-2**	**F-3**	**F-4**	**F-5**
KNO_3	1.25	1.50	1.25	1.50	1.50
KH_2PO_4	1.25	1.25	1.50	1.50	1.50
$MgSO_4.7H_2O$	1.00	1.00	1.00	1.00	1.50
$FeSO_4.7H_2O$	0.0498	0.0498	0.0498	0.0498	0.0498
EDTA	0.50				
H_3BO_3	0.1142				
$CaCl_2.2H_2O$	0.1110				
$ZnSO_4.7H_2O$	0.0882				
$CuSO_4.5H_2O$	0.0157				
$MnCl_2.4H_2O$	0.0142				
MoO_3	0.0071				
$Co(NO_3)_2.6H_2O$	0.0049				

2.2.7 Qualitative secondary metabolite analysis

The secondary metabolite was analysed according to Prabakaran *et al.* (2018) about alkaloids with a comparison solution of quercetin at a wavelength of 500 nm and according to Hazra *et al.* (2008) about flavonoid and phenolic at a wavelength of 500 nm. Total phenolic was calibrated against a gallic acid standard and expressed as mg gallic acid equivalent. Chaudhuri *et al.* (2014) method was followed about tannins at a wavelength of 500 nm. Then, saponin at a wavelength of 430 nm.

2.3 Analysis Data

The experiment was carried out in three repetitions and the results were expressed as the mean ± standard deviation. The experimental results were analyzed using ANOVA, and differences between treatments ($p<0.05$) were carried out using the Duncan test software SPSS version 26.

3. Results and Discussion

3.1 Chlorophyll and Carotenoid Content

The values for the chlorophyll a, chlorophyll b and carotenoid content of *Chlorella vulgaris* in each treatment can be seen in Table 2.

Table 2. Content of chlorophyll a, b, total chlorophyll and carotenoids of *Chlorella vulgaris* with different cultivation media formulations.

Treatments	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Total chlorophyll (µg/mL)	Carotenoid (µg/mL)
F-1	29.41 ± 0.10^{d}	50.29 ± 0.04^{e}	79.70 ± 0.04^{d}	0.01 ± 0.01^{a}
F-2	38.19 ± 0.15^{e}	41.45 ± 0.03^{d}	79.65 ± 0.02^{d}	0.08 ± 0.01^{b}
F-3	15.92 ± 0.21^{a}	11.43 ± 0.04^{a}	27.35 ± 0.04^{a}	1.85 ± 0.02^{e}
F-4	18.58 ± 0.15^{b}	21.95 ± 0.03^{b}	40.53 ± 0.05^{b}	0.79 ± 0.01^{d}
F-5	21.85 ± 0.06^{c}	26.58 ± 0.03^{c}	48.42 ± 0.04^{c}	0.34 ± 0.01^{c}

Note: Data in the same column marked with different manuscripts was significantly different ($p<0.05$)

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoids in F-1, F-2, F-3, F-4, and F-5 showed very significant differences among treatments ($P<0.05$). The highest chlorophyll a was found in the F-2 treatment (38.19 µg/mL), and the highest chlorophyll b was found in the F-1 treatment (50.29 µg/mL), which is significantly different from other treatments (Figure 3). Meanwhile, total chlorophyll in the F-1 and F-2 treatments were not significantly different. The highest carotenoids were found in the F-3 treatment (1.85 µg/mL), which was significantly different from other treatments (Figure 4).

The results of this study have a higher chlorophyll content compared to Mirzaie *et al* (2016) and shows the content of chlorophyll of *Chlorella* at 12 days of cultivation 10 µg/mL, and chlorophyll b 10 µg/mL, while the content of chlorophyll a according to Wu *et al.* (2022) was 30.42 µg/mL; it was almost the same as the content of chlorophyll a of *Chlorella* in F-1 treatment. Overall, the total chlorophyll in this study was higher than *Nannochloropsis* sp (30.59 µg/mL); *Scenedesmus* sp (19.00 µg/mL); *Dunaliella* sp (23.65 µg/mL); and products *Chlorella* sp commercially (10.35 µg/mL); whereas in the same study the total chlorophyll in control treatment was higher than Kent *et al.* (2015) namely 7.96 µg/mL. The high levels of *Chlorella* pigment are shown in Figure 3.

The presence of KNO_3 in *Chlorella* cultivation is thought to increase chlorophyll content. This is according to Coban *et al.* (2021) the administration of KNO_3 compounds functions as a source of nitrogen which is one of the nutrients in the culture media that functions in the formation of chlorophyll so that chlorophyll can increase. However, the increase of KNO_3 combined with KH_2PO_4 and Mg_2SO_4 in cultivation can cause the chlorophyll content to decrease relatively. This is according to the finding by Muhammad *et al.* (2022) that an inappropriate combination of nitrogen and potassium in the planting medium can reduce the accumulation of green leaf pigment (chlorophyll). The decrease in chlorophyll levels in this study suggests that competition and nutritional pressure may be due to the influence of high doses of KNO_3 and KH_2PO_4 in F-3, F-4, and F-5 treatments to interfere with cell growth of *Chlorella* during cultivation. This reason is supported by Peng *et al.* (2017) and Srinivasan *et al.* (2018) that a higher dose in the cultivation medium will increase oxygen pressure by reducing the dCO ra-

tio$_2$ against dO$_2$ to disrupt the balance of photosynthesis and photorespiration. This fact is also influenced by the antioxidant value.

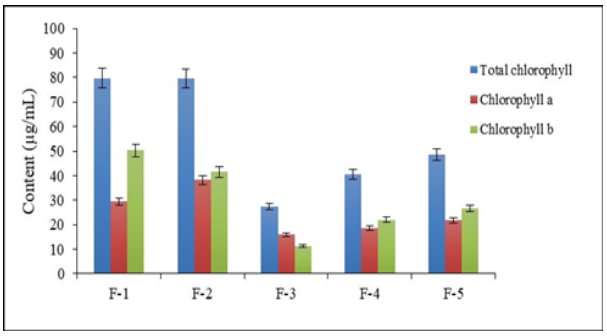

Figure 3. Total content of chlorophyll, chlorophyll a, b of *Chlorella vulgaris.*

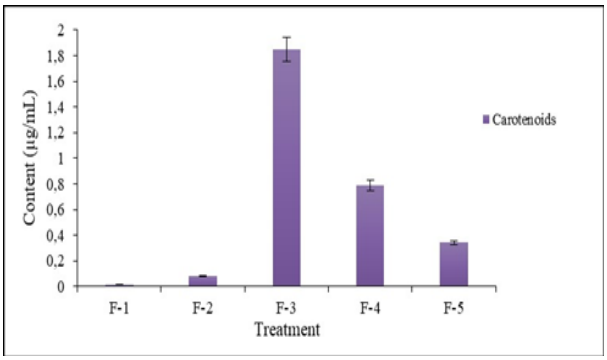

Figure 4. Carotenoids content of *Chlorella vulgaris.*

3.2 Antioxidant Activity

Antioxidant activity showed very significant differences between treatments (P<0.05). F-1 treatment is significantly different from other treatments (F-2, F-3, F-4, and F-5), but has the same strong category as F-2 treatment (Table 3). Antioxidant activity in this study used an inhibitory concentration of 50, which was quantified using linear regression on the inhibitory concentration (ln) of *Chlorella* extract and the DPPH value (inhibition %), resulting in a very strong antioxidant correlation in inhibiting free radicals (Septrianzu, 2024) (Figure 5).

Table 3. Antioxidant activity *Chlorella vulgaris* with different cultivation media compositions.

Treatment	IC$_{50}$ (mg/L)	Criteria*
F-1	88.57 ± 10.94^{c}	Strong
F-2	49.52 ± 6.88^{b}	Strong
F-3	235.95 ± 10.55^{e}	Moderate
F-4	221.27 ± 9.78^{e}	Moderate
F-5	128.88 ± 6.36^{d}	Moderate
Ascorbic acid	2.29 ± 0.23^{a}	Very strong

Note: Data in the same column marked with different manuscripts is significantly different (p<0.01). * Molyneux (2004).

The highest IC$_{50}$ value for *Chlorella* extract is F-2 treatment, followed by F-1, F-5, F-4, F-3, however, it does not match the vitamin C standard yet. Very strong antioxidants show IC$_{50}$ <50 mg/L, moderate antioxidants 100-150 mg/L, weak antioxidants 150-200 mg/L, and very weak antioxidants >200 mg/L (Ridlo *et al.*, 2023). Therefore, *Chlorella* extract in F-2 and F-1 treatment showed strong antioxidant activity.

The value of antioxidants in the F-1 and F-2 treatment in this study is almost the same as in previous research, namely 32.74 mg/L strong category (Iriani *et al.*, 2017). But lower than *Chlorella sorokiniana* according to Olasehinde *et al.* (2017), namely 145.26-146.06 mg/L, and Putri *et al.* (2021), namely 127 mg/L with moderate activity category. In this study, the appropriate KNO$_3$ formulation was found in the F-2 treatment because it affected the high antioxidant content. This reason is also supported by Einali *et al.* (2013) where culture *Dunaliella salina* with different conditions of nitrogen in the form of KNO$_3$ 5 mM affects cell growth and chlorophyll, where high cell density produces high antioxidants compared to low cell density. Chlorophyll can function to protect the human body by neutralizing free radicals (ROS), which attack macromolecules such as lipid membranes, proteins and DNA, which cause cancer, diabetes mellitus, ageing and neurodegeneration in the body (Ngo *et al.*, 2011; Roy *et al.*, 2023). Antioxidants used for ROS detoxification come from enzymatic and non-enzymatic sources by working intracellularly or extracellularly (such as reducing O$_2$ radicals, scavengers, electron donors, hydrogen donors, peroxide degraders, enzyme inhibitors, gene expression regulators, synergists, and metal chelators. agent) (Kurutas, 2016; de Almeida *et al.*, 2022; Sharifi-Rad *et al.*, 2022; Chaudhary *et al.*, 2023). Lower antioxidant activity values (F-3, F-4, and F-5 treatments) indicate the formation of chlorophyll *Chlorella* which, is also low (Table 2) so that it can weaken detoxification in chloroplasts. Therefore, high chlorophyll concentrations in F-1 and F-2 treatments (Table 2) are very necessary for O$_2$ activation in stabilizing triplet oxygen (^{3}O$_2$) (Coulombier *et al.*, 2021). Therefore, the optimal combination is found in the F-2 treatment (1.50 g/L KNO$_3$, 1.25 g/L KH$_2$PO$_4$, 1.0 g/L MgSO$_4$) which is equal to the F-1 treatment.

3.3 Amino Acid

Chlorella vulgaris was cultivated with F-2 formulation containing 17 amino acids (Table 4) with a total amino acid composition is 301.52 mg/g with an essential amino acid composition of 134.49 mg/g and non-essential 167.03 mg/g. This type of essential amino acid is characterized by high levels of leucine, arginine and lysine, namely 28.87 mg/g, 24.54 mg/g,

and 22.76 mg/g, respectively, and the types of non-essential amino acids are characterized by high levels of glutamic acid, aspartic acid and alanine, namely 57.20 mg/g, 31.12 mg/g, and 27.50 mg/g, respectively; more details are shown in Table 4.

Table 4. Amino acid content *Chlorella vulgaris* in dry weight.

Types of amino acids	Amount (mg/g)
Aspartic Acid	31.12
Glutamic Acid	57.20
Serine	9.17
Glycine	16.04
Histidine*	7.05
Arginine*	24.54
Threonine*	9.84
Alanin	27.50
Proline	11.25
Tyrosine	7.86
Valine*	13.44
Methionine*	7.34
Sistein	6.89
Isoleucine*	8.55
Leucine*	28.87
Phenylalanine*	12.10
Lisine*	22.76
Total	301.52

* essential amino acids

Amino acids are the main constituent of microalgae protein in large quantities and are needed by the human body (Siahbalaei *et al*., 2021). The amino acid composition in this study was slightly lower than that of microalgae *Nannochloropsis* namely 302.95 mg/g, *Scenedesmus* namely 309.85 mg/g, and *Dunaliella,* namely 341.68 mg/g, which is characterized by the dominant amino acids leucine, arginine, lysine (essential) and glutamic acid, aspartic acid, alanine (non-essential) compared to other types of amino acids (Kent *et al*., 2015).

Microalgae amino acids are closely related to the formation of sugars and pigments such as chlorophyll through their biochemical composition (Wang *et al*., 2021). The better the photosynthesis process in forming chlorophyll can improve the quality of nutrients such as protein (Brown *et al*., 1993; Barkia *et al*., 2019; Ridlo *et al*., 2023). In addition, the amino acids produced from *Chlorella* are capable of producing recombinant proteins at the same level as microalgae *Chlamydomonas reinhardtii* applied as antibodies, immunotoxins, anticancer, hormones, vaccines, *nutraceutical* intestinal, and therapeutic (Rasala *et al*., 2015; Dubey *et al*., 2023).

3.4 Fatty Acid

Chlorella vulgaris was cultivated with F-2 formulation containing seven fatty acid profiles (Table 5). The total fatty acid composition is 84.32 mg/g, with the saturated fatty acid composition 22.45 mg/g; monounsaturated fatty acids 12.72 mg/g, and unsaturated fatty acids 49.15 mg/g. This type of fatty acid is characterized by high levels of oleic, linoleic and palmitic, namely 24.19 mg/g, 23.64 mg/g, and 21.59 mg/g, respectively. The detailed data are shown in Table 5.

Meanwhile, the total fatty acids of *Chlorella vulgaris* are higher than the *Scendesmus* sp (82.08 mg/g), *Dunaliella* sp., namely 66.32 mg/g, spirulina commercially, namely 48.73 mg/g and *Chlorella* commercial 60.60 mg/g (Kent *et al*., 2015), but lower than *Chlorella vulgaris* 96.6 mg/g (Tokusoglu *et al*., 2003) and *Isochrysis galbana* 104.59 mg/g (Shekarabi *et al*., 2019). Myristic saturated fatty acid is higher than spirulina 0.43 mg/g and *Chlorella* 0.38 mg/g, and palmitic saturated fatty acids are also higher than spirulina 15.41 mg/g and *Chlorella* 6.04 mg/g (Tokusoglu *et al*., 2003; El-Sheekh *et al*., 2020). Moreover, the unsaturated fatty acid oleic is higher in the extract *Chlorella* (16.07 mg/g). The unsaturated fatty acids linoleic and linolenic were also higher than the research extract *Spirulina,* respectively, namely 10.37-11.25 mg/g and 0.62-0.71 mg/g, but linoleic is still lower than *Chlorella* MF1 27.72 mg/g (Tokusoglu *et al*., 2003; El-Sheekh *et al*., 2020). Furthermore, total unsaturated fatty acids (MUFA and PUFA) are higher than *Chlorella* sp. (El-Sheekh *et al*., 2020) namely 45.27 mg/g and compound unsaturated fatty acids (PUFA) higher than *Chlorella* namely 38.94 mg/g, spirulina 22.30-25.13 mg/g, and *Isochrisis* namely 24.41 mg/g (Tokusoglu *et al*., 2003). These fatty acids (PUFA) are precursors of prostaglandins in acids eicosapentaenoic (EPA) and docosahexaenoic (DHA), which have human health effects (Valenzula *et al*., 2011; Ahmad *et al*., 2024). This research proves that a large amount of fatty acids indicates greater potential for the use of functional foods such as biodiesel raw materials, food and feed additives because the total fatty acids are >50% (Stansell *et al*., 2012; Darwish *et al*., 2020). The high levels of amino acids and fatty acids in this study prove the high antioxidant value (Table 4) and secondary metabolite content (Table 6).

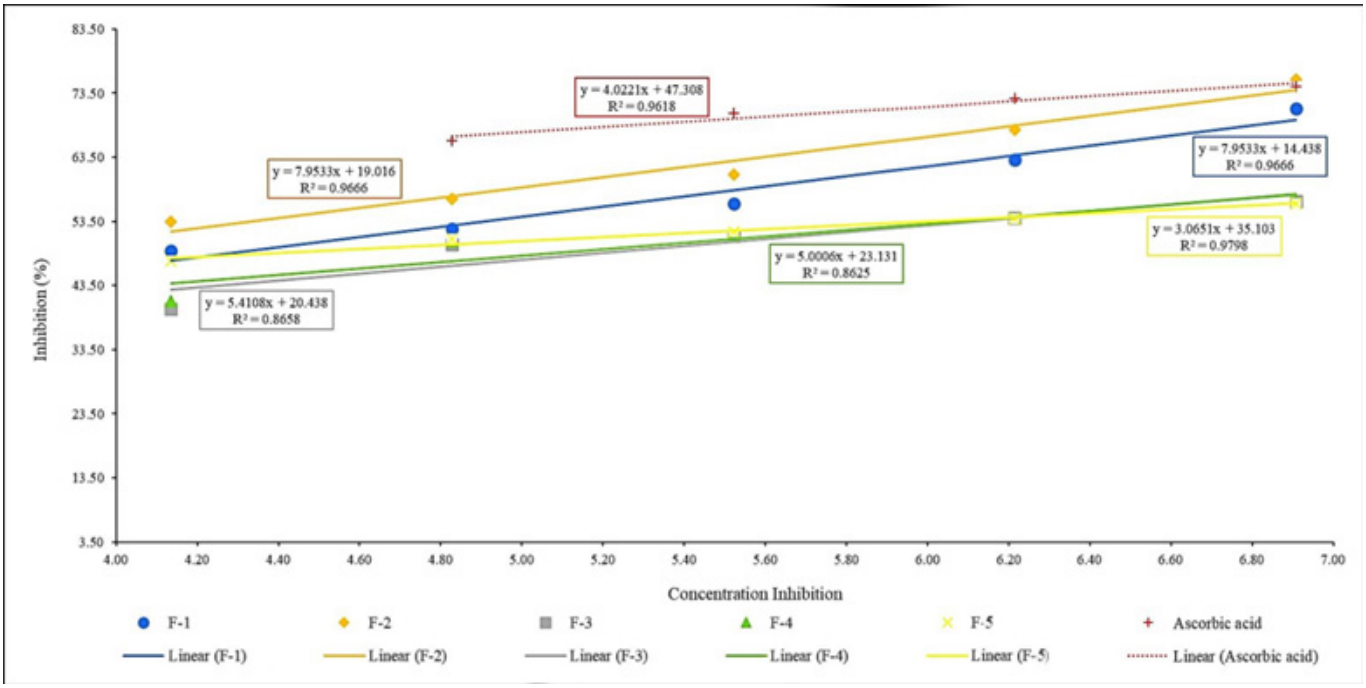

Figure 5. Antioxidant activity graph of *Chlorella vulgaris* in different formulation media.

Table 5. Fatty acid content *Chlorella vulgaris* in dry weight.

Fatty Acids	mg/g	Saturated and Unsaturated fatty acid (mg/g)
Lauric[1]	0.30	
Myristic[1]	0.56	22.45
Palmitic[1]	21.59	
Stearic[2]	12.72	12.72
Oleic[3]	24.19	
Linoleic[3]	23.64	49.15
Linolenic[3]	1.32	
Total		84.32

1 saturated fatty acids (SFA)
2 monounsaturated fatty acids (MUFA)
3 polyunsaturated fatty acids (PUFA)

Table 6. Secondary metabolite content *Chlorella vulgaris* in 100 g.

Type	Mg/L
Alkaloid	109.471
Flavonoid	82.111
Saponin	1.342.222
Tannin	411.591
Phenolic	151.889

3.5 Secondary Metabolite

The *Chlorella vulgaris* was cultivated with F-2 formulation containing secondary metabolites including alkaloids, flavonoids, saponins, tannins, and phenolic. The highest secondary metabolite is saponin, with a value of 1342.222 mg⁻L and the lowest is flavonoid 82.111 mg⁻L; more details are shown in Table 6.

In this study, the secondary metabolites content was not much different from *Chlorella vulgaris,* which was reported by Pradhan *et al.* (2020) who obtained secondary metabolite covering alkaloid, flavonoid, saponin, soil, phenolic, and steroid. Phenolic compound in this study was higher than microalgae *Chlorella vulgaris* 43-45 mg/L (Jerez-Martel *et al.,* 2017), *Isochrysis galbana Parke, Tetraselmis chuii, Dunaliella salina, Teodoresco* namely a maximum of 17,798 mg/L (Widowati *et al.*, 2021); *Euglena cantabrica* namely a maximum of 2.97 mg/L (Jerez-Martel *et al.,* 2017); and flavonoid compounds higher than *Scenedesmus obliquus,* namely 66.56 mg/L (El-Chaghaby *et al.,* 2019), but lower than *Chlorella vulgaris,* namely 470-549 mg/L (Chaudhuri *et al.,* 2014; El-Chaghaby *et al.,* 2019; Pradhan *et al.,* 2020). Increased secondary metabolites such as phenolic and flavonoid are suspected to be closely related to increased growth, which is directly proportional to the level of chlorophyll.

Phenolic, saponin, and alkaloid compounds in this research are the most important compounds in algal genera because it has a good effect on the health

of the human body with a defence mechanism against endogenous and exogenous environmental effects, such as oxidative processes, light, temperature and pathogen invasion (De Souza *et al*., 2019; Pradhan *et al*., 2020). Nafiu and Ashafa (2017), Coulombier *et al.* (2021), and Vignaud *et al.* (2023) reported that this compound has antioxidant properties due to the large number of reactive constituents which increase the inhibitory power of free radicals by >50%. Mandal *et al.* (2005) reported the phenolic and saponin compounds in *Chlorella* have biological functions such as hypercholesterolemia, antioxidant, anticancer, anti-inflammatory, and as a significant weight loss drug. In addition, flavonoid and tannin in *Chlorella* have antimicrobial, antioxidant, spasmolytic and α-glucosidase inhibitory activity (Polterait, 1997; Pandithurai *et al*., 2015; Dinev *et al*., 2021). It has been proven that flavonoid compounds can provide great inhibitory power to *Escherichia coli* (Pradhan *et al*., 2020). Therefore, the secondary metabolite content of *Chlorella* in this study is of great interest for the formulation of new drugs in the therapeutic use of the macro mineral nitrogen (KNO_3) (Sassi *et al*., 2020).

The findings of this study indicate that the formula cultivation of *Chlorella vulgaris* in the F-2 treatment with formulation (g L^{-1}) 1.50 KNO_3, 1.25 KH_2PO_4, 1,0 $MgSO_4.7H_2O$, 0.0498 $FeSO_4.7H_2O$ is the best treatment which has an effect on chlorophyll, carotenoids and has antioxidant activity in the strong category. The use of F-2 formulation can replace the commercial media of *Chlorella* cultivation. Furthermore, it also has a good amino acid, fatty acid and secondary metabolite profile. Therefore, F-2 treatment can be used as a formulation in cultivation for large-scale production of *Chlorella*, and could be applied as a functional food or health supplement.

4. Conclusion

Chlorella vulgaris offers significant health benefits due to its high chlorophyll content and antioxidant properties. Optimizing its cultivation by enhancing nutrient factors such as nitrogen, phosphorus, and magnesium can improve its nutritional value and antioxidant quality. Future research should explore and investigate its application as a functional food or pharmaceutical raw material.

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Authors' Contributions

The contributions of each author as follow, Dian Iriani; devised the main conceptual ideas, collected the data and drafted the manuscript. Feliatra, Bustari Hasan; devised the main conceptual ideas and critical revision of the article. Rahman Karnila; A data analysis and critical revision of the article. Nittaya Chaiyanate: devised the main conceptual ideas. Rozi: article revision.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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