https://doi.org/10.48048/tis.2025.9219

Enhancement of Arachidonic Acid Production by A Newly Isolated *Diplosphaera* sp. Associated with Bryophyte

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Received: 25 October 2024, Revised: 25 November 2024, Accepted: 2 December 2024, Published: 20 January 2025

Abstract

Arachidonic acid (ARA) is an essential omega-6 polyunsaturated fatty acid with significant roles in nervous system development and various physiological processes. While ARA production from freshwater microalgae has been limited, this study aimed to optimize cultivation conditions for ARA production in *Diplosphaera* sp. DMKU4.11.2, a newly isolated strain from a bryophyte in Thailand. Among 6 tested media, BG11 demonstrated the highest ARA production. Further optimization of the BG11 medium involved adjusting factors such as the nitrogen source, nitrogen concentration, phosphorus ratio, phosphorus level and sodium chloride supplementation. The optimal conditions were determined to be BG11 medium containing 0.75 g/L sodium nitrate and potassium phosphate at a 1:1 ratio, with 3.50 mg P/L. Under these conditions, *Diplosphaera sp.* DMKU4.11.2 produced the highest ARA yield of 115.31 ± 2.03 mg/g dry cell weight (DCW), with an ARA production rate of 3.23 ± 0.12 mg/L/day. Supplementing the medium with 50 mM sodium chloride further enhanced ARA content to 9.57 ± 1.67 % of total fatty acids (TFAs) and increased biomass production to 0.36 ± 0.00 g/L. Culturing in the optimized BG11 medium resulted in a 1.24-fold increase in growth and a 3.95-fold increase in ARA content compared to the standard BG11 medium. In conclusion, this study highlights the potential of *Diplosphaera* sp. DMKU4.11.2 as a sustainable source for ARA production, with opportunities to enhance productivity for industrial and health-related applications.

Keywords: Arachidonic acid, Polyunsaturated fatty acids, Microalgae, *Diplosphaera* sp., Optimization

Introduction

Arachidonic acid (ARA, C20:4(n-6)) is an essential polyunsaturated omega-6 fatty acid that an essential role in human physiology, serving as a key component of brain cell phospholipids and a precursor to bioactive molecules such as prostaglandins and leukotrienes [1,2]. ARA is important for growth, development, and reproductive health in mammals and aquatic organisms, playing a crucial role in maintaining the integrity of cell membranes [3,4]. Since mammals, including humans, cannot synthesize ARA, it must be obtained through dietary sources such as fish, seeds, oils and meat, or via the metabolic conversion of linoleic acid (LA), primarily derived from plants, microbes and fish. Previously, the oleaginous fungi such as *Mortierella alpina* have been extensively studied and utilized for ARA production due to their well-characterized biosynthetic pathways. However, the high costs associated with large-scale fermentation limit their feasibility for widespread commercial application [5,6]. Similarly, other conventional sources face challenges related to sustainability, high production costs and variability in ARA content. However, in this context, microalgae have emerged as a promising and sustainable alternative for ARA production [7,8].

Microalgae present several advantages over conventional ARA sources. Unlike higher plants, which lack the enzymes needed to produce significant amounts of long-chain polyunsaturated fatty acids (PUFAs) such

as ARA and DHA, certain microalgal species can efficiently biosynthesize and accumulate these essential fatty acids. Additionally, microalgae utilize simple minerals and CO_2 to generate biomass while producing high-value PUFAs like ARA [9-12], making them particularly appealing for climate change mitigation and sustainable biomanufacturing. These unique characteristics position microalgae as an environmentally sustainable and renewable source of ARA, offering a significant advantage in addressing global nutritional demands.

However, the selecting appropriate microalgal strains, optimizing nutrient composition, and refining cultivation durations are critical for achieving high biomass and lipid yields [13]. Several marine microalgae are known for their ability to produce ARA, such as *Porphyridium cruentum*, a red microalga capable of accumulating significant amounts of ARA and other PUFAs [14]. *Nannochloropsis sp.* is another well-researched genus that produces ARA along with eicosapentaenoic acid (EPA) [15]. Although marine microalgae have been well-studied for their ARA and PUFAs production, there is limited research on freshwater microalgae, especially those isolated from bryophytes.

Bryophytes are key components of aerial habitats and serve as important microhabitats for algae. Their structures, such as overlapping leaves, leaf bases, paraphyllia, papillae and rhizoids, trap and retain water, creating a stable environment that supports algal growth. The moisture-holding capacity of bryophytes is critical for sustaining algal communities, making these habitats an excellent source for exploring potential ARA-producing microalgae [16,17]. For example, certain microalgae, such as *Parietochloris incisa*, have been identified as capable of accumulating ARA-rich triacylglycerols (TAG), indicating their potential as ARA sources [18,19]. However, the role of freshwater microalgae in ARA production remains largely unexplored.

Therefore, this study aims to investigate freshwater microalgae isolated from bryophytes in Thailand as potential new sources for ARA production. The subsequent step involves optimizing several factors, including the culture medium, nitrogen and phosphorus sources, their respective concentrations and stressors such as salinity, to enhance biomass and ARA production. By targeting specific freshwater strains capable of high ARA production, this research contributes to the sustainable production of this essential fatty acid and expands the pool of available alternatives for aquaculture and human nutrition.

Materials and methods

Sample collection and microalgae isolation

The samples of Bryophyte were collected from national parks, waterfalls and water reservoirs in the northern, northeastern, central and southern regions of Thailand. A total of 23 sampling sites were used to isolate the microalgae. The samples were washed with a 0.85 % sodium chloride solution, ground in a mortar, and filtered through a 256 µm plankton net. The filtered sample was subsequently spread onto a C medium plate [20] containing nalidixic acid, cefotaxime, ampicillin, chloramphenicol, thiabendazole and sodium propionate to eliminate bacterial and fungal contamination. Single colonies were picked and streaked onto C agar plates to obtain pure cultures. The microalga was cultured in C medium and incubated at 25 ± 2 °C under a light intensity of 40 µmol/m^2/s by cool-white fluorescent lamps with a 16:8 h, light: dark photoperiod for 14 days. Cultures were maintained on C agar and sub-cultured every 2 weeks.

Screening of ARA-producing microalgal strain

Screening for ARA production was conducted in 3 stages. In the primary screening, microalgae were cultured in 15 mL of C broth and incubated at 25 °C on an incubator shaker at 120 rpm for 14 days to select microalgae capable of producing arachidonic acid. The ARA-producing isolates were then subjected to secondary and tertiary screenings. In the secondary and tertiary screenings, the isolated microalgae were cultured in 300 mL Erlenmeyer flasks containing 170 mL of C medium, with 3 replicates. The initial optical density at 600 nm was set to 0.2, with shaking at 150 rpm under a light intensity of 40 µmol/m²/s and a 16:8 h light-dark photoperiod. For secondary screening, isolates were selected based on their ability to produce ARA at concentrations exceeding 40 mg/L. In the tertiary screening, the cultures were maintained at 2 different temperatures, 18 and 25 °C. The microalgae were evaluated for their ability to produce ARA under stress conditions for further experiments.

Identification of microalgal strain

The microalgae strain was initially identified using a light microscope, followed by molecular characterization through 18S rRNA sequence analysis. The microalgae were then cultivated in BG11 broth and incubated at 25 ± 2 °C for 10 days in an incubator shaker. Cells were collected, and DNA was extracted following the method of Doyle and Doyle [21]. PCR amplification of the isolated microalgal DNA was performed using the universal eukaryotic primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by White *et al.* [22]. PCR amplification was performed using a thermal cycler (Perkin Elmer GeneAmp PCR System 2400) with the following program: Initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 58.1 °C for 45 s and extension at 72 °C for 1 min. After completing the cycles, an elongation step was performed at 72 °C for 5 min. PCR products were analyzed by 1 % agarose gel electrophoresis in 1X TAE buffer and compared with a DNA marker (1 kb plus DNA ladder). These primers target the internal transcribed spacer (ITS) region of ribosomal DNA, encompassing the ITS1 and ITS2 regions along with the 5.8S gene. The PCR product sequences were analyzed using NCBI BLASTn (http://www.ncbi.nlm.nih.gov), and a phylogenetic tree was constructed with MEGA version 11 (http://www.megasoftware.net).

Optimization of cultivation conditions for growth and ARA production

Effects of the types of culture media

Six different types of culture media were investigated, namely, AF 6 [23], BBM [24], BG11 [25], Modified Chu 13 [26], C [20] and NS III media [27]. The green microalga *Diplosphaera* sp. DMKU4.11.2 was cultured in 150 mL of culture media in 250 mL conical flasks and then incubated on a rotary shaker at 150 rpm, 25 ± 2 °C under a 16:8 h light: dark photoperiod for 10 days.

Effects of nitrogen sources

The green microalga *Diplosphaera* sp. DMKU4.11.2 was cultivated in BG11 medium supplemented with 3 different nitrogen sources: Sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) and urea (CH_4N_2O). Sodium nitrate is typically a component in the standard BG11 medium. The nitrogen concentration from each source was standardized to 247 mg N/L, equivalent to that in the standard BG11. The microalga was cultivated in a 200 mL conical culture tube (4×30 cm^2) containing 150 mL of the culture medium and incubated at 25 ± 2 °C under a light intensity of 40 µmol/m^2/s with a 24:0 h photoperiod, and 0.1 vvm of bubbling air. Samples were collected every 3 days for 15 days. These culture conditions were used in all experiments.

Effects of nitrogen levels

The selected nitrogen source was investigated at different levels of 0.00, 61.77, 123.54, 185.31, 247.07 and 308.84 mg N/L (equivalent to 0.00, 0.38, 0.75, 1.13, 1.50 and 1.88 g/L) in the BG11 medium.

Effects of phosphorus ratios

The green microalga *Diplosphaera* sp. DMKU4.11.2 was cultivated in a modified BG11 medium containing 2 phosphorus sources: Dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4). These phosphorus sources were mixed in different ratios (100:0, 75:25, 50:50, 25:75 and 0:100) while maintaining a total phosphorus concentration of 7.00 mg P/L. The standard BG11 medium contains only dipotassium hydrogen phosphate with a phosphorus concentration of 7.00 mg P/L.

Effects of phosphorus levels

The selected phosphate ratio was investigated at various phosphorus levels of 0.00, 1.75, 3.50, 5.25, 7.00 and 8.75 mg P/L in the BG11 medium.

Effects of sodium chloride

The green microalga *Diplosphaera* sp. DMKU4.11.2 was cultivated in a modified BG11 medium supplemented with 6 different concentrations of sodium chloride: 0, 50, 100, 150, 200 and 400 mM.

Analytical method

Determination of biomass concentration

The growth of green microalga *Diplosphaera* sp. DMKU4.11.2 was analyzed by measuring the optical density at 600 nm using a spectrophotometer (UV-

PharmaSpec 1700). The biomass concentration (g/L) was calculated using the following formula:

$$\text{Biomass concentration (g/L)} = (0.2198 \times OD_{600}) - 0.013 \ (R^2 = 0.9986).$$

Fatty acid extraction and fatty acid methyl esters (FAME) analysis

The *Diplosphaera* sp. DMKU4.11.2 cells were harvested by centrifugation at 3,300 rpm for 15 min and washed twice with deionized water. Subsequently, 0.5 mL of distilled water and 100 µL of the internal standard, pentadecanoic acid (C15:0), were added to the sample and mixed thoroughly. The sample was extracted using 3.75 mL of a chloroform-methanol mixture (1:2 ratio). Lipid extraction followed a modified method by Bligh and Dyer [28]. Transmethylation of the lipid was carried out using 6 % sulfuric acid in methanol at 80 °C for 15 h [29,30]. The resulting fatty acid methyl esters were analyzed by gas chromatography (GC-2010 Plus; Shimadzu, Tokyo, Japan). ARA was determined by comparing the retention time to that of an authentic ARA standard (Sigma-Aldrich).

Statistical analysis

The average values and standard deviations of the biomass and lipid content from 3 replicates were calculated. Significant differences were determined using analysis of variance (ANOVA) at a 95 % confidence interval ($p < 0.05$).

Results and discussion

Isolation and screening of ARA-producing microalgae

Bryophyte samples were collected from 23 locations, including national parks, waterfalls and various water sources across northern, central and southern regions of Thailand, for microalgae isolation. These environments were selected due to their diverse microalgal communities and minimal human disturbance. Additionally, the regions offered varied light exposure and nutrient availability, which are important factors for microalgal growth [31]. A total of 94 microalgae isolates were obtained for the ARA production screening experiment.

In the primary screening, all 94 isolates were tested, with 38 identified as ARA producers. In the secondary screening, 8 isolates demonstrated ARA production at concentrations exceeding 40 mg/L: DMKU2.1.1, DMKU4.6.1, DMKU4.11.2, DMKU4.28, DMKU4.32, DMKU4.39, DMKU7.16 and DMKU9.7. These isolates were further assessed in the tertiary screening to evaluate their ability to produce ARA under stress conditions, specifically low-temperature cultivation. Among them, the isolate DMKU4.11.2 was selected for further study due to its high growth rate and ARA production capacity of 0.69 ± 0.01 g/L and 109.87 ± 5.10 mg/g of DCW, respectively (**Table 1**). The standard deviation reflects the consistency of ARA production, while *p*-values indicate the statistical significance of differences between experimental groups. Low standard deviations paired with significant *p*-values ($p < 0.05$) suggest reliable and robust results. In contrast, high standard deviations and non-significant *p*-values may point to variability in metabolic responses or inconsistencies in experimental conditions. Moreover, this isolate showed the potential for 1.23-fold increase ARA production under stress conditions compared to normal conditions.

Table 1 Screening of ARA-producing microalgae isolated from bryophytes.

Isolates	Culture condition							
	Normal condition (25 °C)				Stress condition (18 °C)			
	DCW (g/L)	Content (% of TFAs)	Production (mg/g)	Productivity (mg/L/day)	DCW (g/L)	Content (% of TFAs)	Production (mg/g)	Productivity (mg/L/day)
DMKU2.1.1	$0.38^{bc} \pm 0.02$	$1.87^{b} \pm 0.07$	$59.79^{abc} \pm 16.74$	$1.61^{ab} \pm 0.38$	$0.58^{c} \pm 0.01$	$2.85^{c} \pm 0.48$	$43.54^{a} \pm 4.47$	$1.67^{ab} \pm 0.03$
DMKU4.6.1	$0.42^{c} \pm 0.01$	$1.86^{b} \pm 0.33$	$68.94^{abc} \pm 12.62$	$2.07^{abc} \pm 0.45$	$0.39^{a} \pm 0.09$	$1.92^{a} \pm 0.01$	$85.59^{b} \pm 5.14$	$2.37^{b} \pm 0.42$
DMKU4.11.2	$0.52^{e} \pm 0.01$	$1.86^{b} \pm 0.42$	$89.50^{bcd} \pm 28.55$	$3.31^{bcd} \pm 0.97$	$0.69^{d} \pm 0.01$	$2.69^{c} \pm 0.27$	$109.87^{d} \pm 5.10$	$5.06^{d} \pm 0.37$
DMKU4.28	$0.40^{c} \pm 0.01$	$1.46^{ab} \pm 0.41$	$38.82^{ab} \pm 14.68$	$1.11^{a} \pm 0.44$	$0.44^{ab} \pm 0.03$	$1.78^{a} \pm 0.19$	$45.66^{a} \pm 1.28$	$1.45^{a} \pm 0.06$
DMKU4.32	$0.32^{a} \pm 0.02$	$0.73^{a} \pm 0.21$	$23.82^{a} \pm 1.13$	$0.54^{a} \pm 0.07$	$0.50^{bc} \pm 0.01$	$2.11^{ab} \pm 0.11$	$111.82^{d} \pm 9.09$	$4.08^{c} \pm 0.38$
DMKU4.39	$0.54^{ef} \pm 0.02$	$1.96^{b} \pm 0.14$	$98.52^{cd} \pm 2.49$	$3.79^{cd} \pm 0.08$	$0.68^{d} \pm 0.05$	$1.88^{a} \pm 0.03$	$103.95^{cd} \pm 0.73$	$4.23^{cd} \pm 0.23$
DMKU7.16	$0.48^{d} \pm 0.01$	$3.17^{c} \pm 1.01$	$126.68^{d} \pm 52.34$	$4.33^{d} \pm 1.88$	$0.74^{d} \pm 0.03$	$1.78^{a} \pm 0.04$	$94.91^{bc} \pm 4.66$	$4.53^{cd} \pm 0.27$
DMKU9.7	$0.35^{ab} \pm 0.03$	$1.29^{ab} \pm 0.14$	$49.74^{abc} \pm 4.29$	$1.23^{a} \pm 0.01$	$0.45^{ab} \pm 0.01$	$2.47^{bc} \pm 0.07$	$129.82^{c} \pm 8.39$	$4.13^{c} \pm 0.40$

Each value represents the mean ± standard deviation. In each column, different letters above paired values indicate that the values obtained from analysis of variance are significantly different ($p < 0.05$).

Morphological and molecular identification of ARA-producing microalgae strain

The morphological analysis showed unicellular green microalgae. The cells of the strain are spherical in shape, with chloroplasts positioned longitudinally near the cell wall (**Figure S1**). The 18S rRNA sequences of isolate 4.11.2 were aligned using Clustal W and compared with other sequences in GenBank using the BLASTN, with *Desmococcus spinocystis* (MK005074.1) used as an outgroup. A phylogenetic tree was then constructed using the Neighbor-Joining method in MEGA version 11.0 (**Figure 1**). The analysis revealed that isolate 4.11.2 is closely related to *Diplosphaera chodati* UTEX 1177 (accession number: HQ129931.1), with a sequence similarity of approximately 96.28 %. Despite this high similarity, the overall identity figures were relatively low, indicating that isolate 4.11.2 may represent a novel species. As a result, isolate 4.11.2 has been renamed *Diplosphaera* sp. DMKU4.11.2.

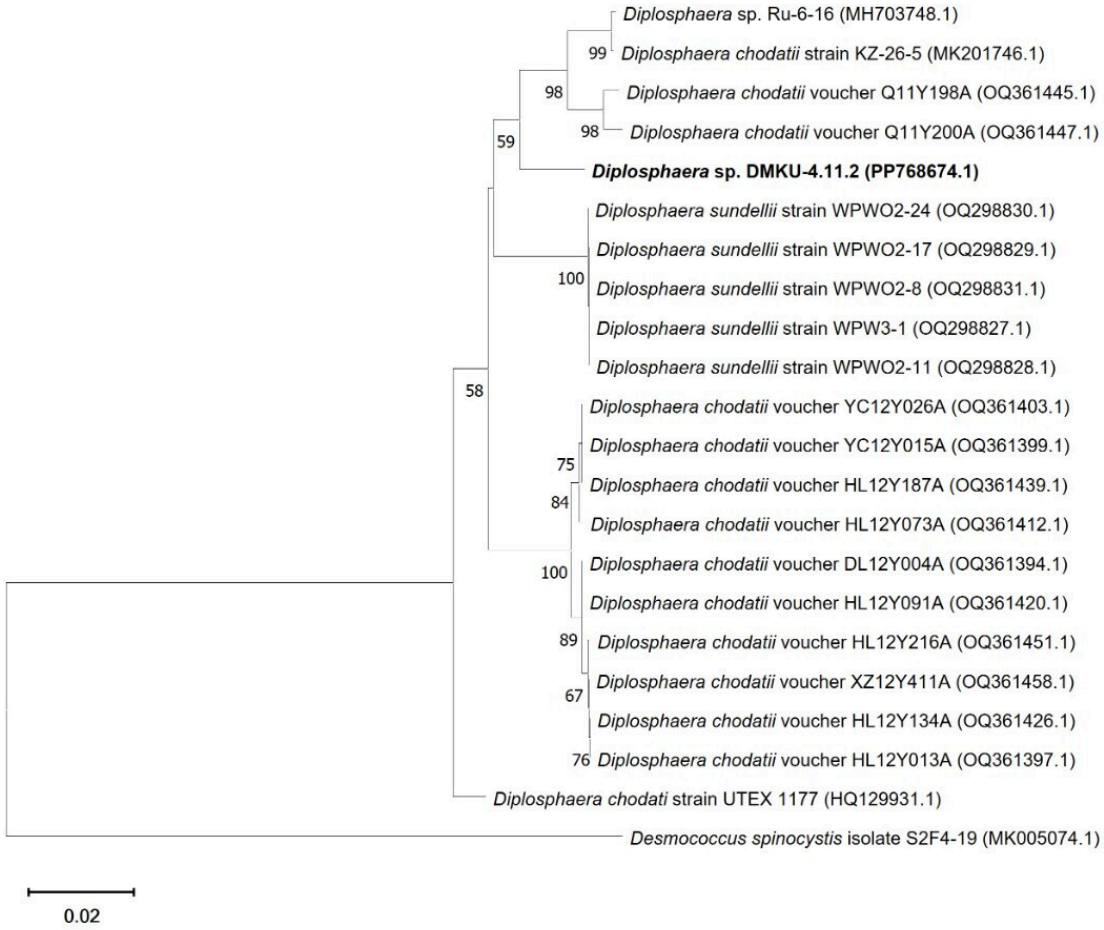

Figure 1 Phylogenetic tree of microalgal isolate 4.11.2, based on ITS sequence, constructed using the neighbor joining algorithm implemented in the MEGA11 software. Bootstrap values below 50 are not displayed. The numbers on the nodes represent bootstrap values as percentages after 1,000 replicates.

Effects of types of culture media on growth and ARA production

The cultivation of microalga *Diplospheara* sp. DMKU4.11.2 in AF 6, BBM, BG11, Modified Chu 13, C and NSIII media revealed that the maximum biomass was obtained in NSIII medium (0.22 ± 0.01 g/L), followed by BG11 (0.20 ± 0.00 g/L), C (0.19 ± 0.00 g/L), BBM (0.18 ± 0.00 g/L), AF6 (0.17 ± 0.01 g/L) and Modified Chu13 (0.16 ± 0.00 g/L) media after 10 days (**Table 2**, **Figure 2(A)**). Notably, NSIII medium provided significantly higher biomass compared to other media, possibly due to its high nitrogen and phosphorus concentrations of 10 and 3.39 mM, respectively. Moreover, NSIII contained 2 types of phosphorus sources, resulting in a higher total phosphorus level than BG11 medium. Muñoz and Guieysse [32] reported that microalgae can utilize high concentrations of nitrogen and phosphorus to synthesize proteins and nucleic acids, contributing up to 40 - 60 % of cell dry weight. Additionally, George *et al*. [33] found that a freshwater microalga *Ankistrodesmus falcatus* grown in BG11 medium exhibited the highest growth rate (0.09 μ/day), surpassing that in BBM medium (0.04 μ/day). The lower growth rate observed in BBM medium may be attributed to its lower nitrogen level, as BBM medium contains 0.25 g/L sodium nitrate [24], compared to BG11 medium with a sodium nitrate level of 1.5 g/L [25].

Table 2 Dry cell weight and ARA production of *Diplosphaera* sp. DMKU4.11.2 cultivating in various media for 10 days.

Media	DCW (g/L)	Arachidonic acid (20:4)		
		Content (% of TFAs)	Production (mg/g of DCW)	Production rate (mg/L/day)
AF6	$0.17^{a} \pm 0.01$	$2.13^{bc} \pm 0.03$	$57.87^{bc} \pm 1.76$	$0.68^{a} \pm 0.18$
BBM	$0.18^{b} \pm 0.00$	$2.57^{cd} \pm 0.06$	$52.53^{b} \pm 5.46$	$0.80^{b} \pm 0.10$
BG11	$0.20^{b} \pm 0.00$	$2.78^{d} \pm 0.32$	$123.19^{d} \pm 12.00$	$2.02^{d} \pm 0.23$
Modified Chu 13	$0.16^{a} \pm 0.00$	$0.92^{a} \pm 0.01$	$25.77^{a} \pm 1.91$	$0.34^{a} \pm 0.03$
C	$0.19^{b} \pm 0.00$	$1.89^{b} \pm 0.22$	$41.37^{ab} \pm 9.59$	$0.65^{a} \pm 0.15$
NSIII	$0.22^{c} \pm 0.01$	$2.78^{d} \pm 1.63$	$76.04^{c} \pm 14.80$	$1.42^{c} \pm 0.20$

DCW: Dry cell weight; TFAs: Total fatty acids. Each value represents the mean ± standard deviation. In each column, different letters above paired values indicate that the values obtained from analysis of variance are significantly different ($p < 0.05$).

Furthermore, it was observed that *Diplospheara* sp. DMKU4.11.2 grown in BG11 medium exhibited the highest ARA production at 123.19 ± 12.00 mg/g of DCW, ARA content of 2.78 ± 0.32 % of TFAs and higher ARA productivity at 2.02 ± 0.23 mg/L/day compared to other media after 10 days. Conversely, NSIII medium produced 76.04 ± 14.80 mg/g of DCW, ARA content of 2.78 ± 1.63 % of TFAs and ARA production rate of 1.42 ± 0.20 mg/L/day. The lowest ARA content of 0.92 ± 0.01 % of TFAs was recorded in cells cultured in Modified Chu 13 medium. The results showed that BG11 produced the highest ARA, which was significantly different from other media at the 95 % confidence level ($p < 0.05$) (**Table 2**, **Figure 2(B)**). In addition, BG11 medium also produced high polyunsaturated fatty acids, γ-linolenic acid 27.40 ± 1.29 % of TFAs and linoleic acid 21.84 ± 0.50 % of TFAs (**Table 4**).

The results indicate that BG11 medium had the highest N:P ratio among the tested media. According to the study by Rasdi and Qin [34], the biomass of *Nannochloropsis oculata* gradually increased with an increase of N:P ratio from 5:1 to 120:1. A high N:P ratio typically signifies phosphorus deficiency for microalgal growth, leading to alterations in lipid biosynthetic pathways that favor lipid accumulation. However, the growth and lipid production of microalgae depend not only on the quantity of macronutrients but also on micronutrients like iron and manganese [35]. BG11 contains higher levels of essential micronutrients compared to other media, which can enhance enzymatic activities critical for lipid biosynthesis. These micronutrients serve as catalytic cofactors for numerous enzymes, precursors for vitamins, and structural components of the cell membrane, all of which are vital for cellular metabolism and lipid synthesis [36]. Specifically, the micronutrients provided by BG11, including H_3BO_3, $MnCl_2$, $ZnSO_4$, $Co(NO_3)_2$ and $NaMoO_4$, have a positive influence on both growth and ARA production in the microalgal strain. These elements support metabolic activities by maintaining cellular homeostasis and activating enzymes in fatty acid elongation and desaturation. While an excess of certain heavy metals can lead to the production of reactive oxygen species (ROS), chlorophyll synthesis inhibition and disruptions in cell proliferation [37], controlled levels of micronutrients, as found in BG11, enhance lipid biosynthesis without causing oxidative stress. Therefore, BG11 medium was selected as a suitable growth and ARA production medium for further studies.

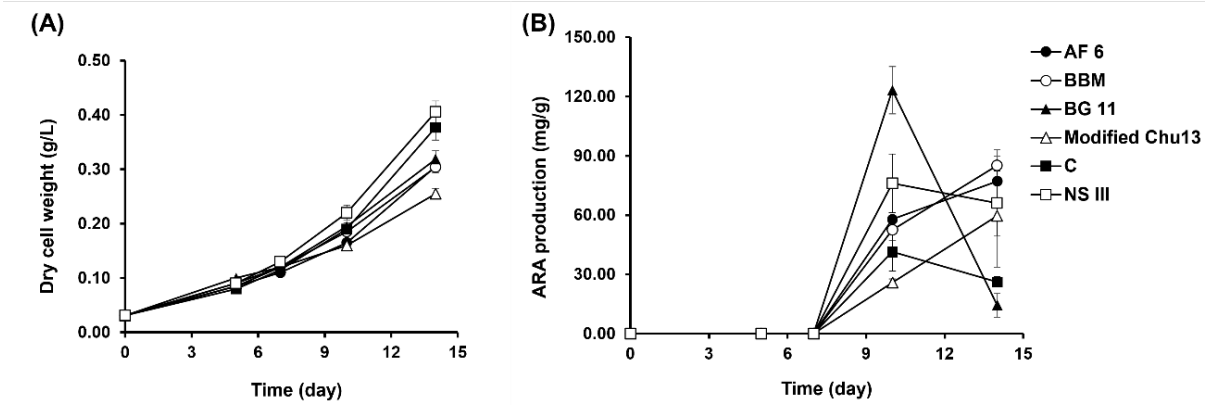

Figure 2 Comparison of the growth and arachidonic acid production of *Diplosphaera* sp. DMKU4.11.2 in various culture media. (A) Growth and (B) ARA production; data are means ± standard deviation of 3 replicate cultures per variant.

Effects of nitrogen sources on growth and ARA production

Nitrogen is a crucial element in the composition of microalgal cell, with microalgae capable of utilizing both inorganic nitrogen (ammonia, nitrate and nitrite) and organic nitrogen (urea, casein and amino acids) sources. Different nitrogen sources exert distinct effects on the physiological indices of microalgae due to their varied utilization pathways [38]. To investigate the effect of nitrogen sources on the growth and ARA production in *Diplosphaera* sp. DMKU4.11.2, 3 different nitrogen sources were tested in BG11 medium, namely sodium nitrate, ammonium chloride and urea. The results showed that *Diplosphaera* sp. DMKU4.11.2 could not grow or produce ARA in the medium containing urea.

Sodium nitrate provided the highest biomass at 0.29 ± 0.03 g/L with a growth rate 0.02 ± 0.00 g/L/day (**Table 3**, **Figure 3(A)**). In addition, sodium nitrate produced the highest ARA 95.70 ± 7.25 mg/g of DCW, and ARA production rate of 2.73 ± 0.08 mg/L/day with an ARA content of 2.42 ± 0.33 % of TFAs. Followed by ammonium chloride had a biomass of 0.22 ± 0.02 g/L and produced ARA of 87.05 ± 8.40 mg/g of DCW with an ARA production rate of 1.91 ± 0.03 mg/L/day. ARA production rate of sodium nitrate was significantly different from other nitrogen sources ($p < 0.05$) (**Tables 3** and **4**, **Figure 3(B)**). However, a closer examination of replicates reveals minor variability, particularly in ARA production, with replicate 2 consistently showing slightly lower ARA yields compared to the others. This may be attributed to potential microenvironmental fluctuations, such as nutrient distribution or minor differences in culture conditions. These findings are consistent with previous research that highlights the effectiveness of nitrate as a nitrogen source for promoting arachidonic acid (ARA) production. For instance, Lourenco *et al.* [39] reported that *Nannochloropsis oculata* produced high levels of ARA when supplied with nitrate. Similarly, Arumugam *et al.* [40] demonstrated that nitrate is the most effective nitrogen source for *Scenedesmus bijugatus*, leading to a significant increase in total lipid content of up to 35 %. Additionally, *Chlorella* sp. HQ was found to produce higher lipid content when grown with nitrate compared to other nitrogen sources [41]. In contrast, nitrogen sources such as urea have been shown to be less effective in promoting ARA production in certain species. For example, Campos *et al.* [42] reported urea as an efficient nitrogen source for the growth of *Nannochloropsis salina*. Moreover, Xu *et al.* [43] found that *Ellipsoidion* sp. showed significantly higher growth rates when cultivated in a medium containing ammonium chloride as the nitrogen source. This variability may be attributed to differences in the genera of microalgae and regulatory genes related to metabolism [44]. The BG11 medium with sodium nitrate, as the standard BG11 medium, which demonstrated the highest biomass and ARA production. Based on these results, it was chosen for further investigation.

Effects of nitrogen levels on growth and ARA production

The optimal level of selected nitrogen sources was investigated at different nitrogen concentrations of

sodium nitrate. It was found that the biomass concentration decreased with increasing nitrate concentration, suggesting that high concentration of sodium nitrate may inhibit the growth of *Diplosphaera* sp. DMKU4.11.2. The highest biomass concentration of 1.13 ± 0.04 g/L was observed in BG11 with 61.77 mg N/L $NaNO_3$ at 15 days, while the lowest biomass concentration of 0.10 ± 0.02 g/L occurred in BG11 with 308.84 mg N/L $NaNO_3$ (**Figure 3(C)**). The results indicate that the optimum nitrogen concentration can enhance the growth of microalgae, while the inappropriate initial nitrogen concentration caused the growth to decline. These results are consistent with the research of Arumugam *et al.* [40], which determined that low concentrations of nitrate (5 - 10 mM) are optimal for cultivating *Scenedesmus bijugatus*. Additionally, *Scenedesmus acuminatus*, *Scenedesmus obliquus* and *Chlorella vulgaris* have demonstrated that these microalgae thrive under limited nitrogen conditions (7.2 - 10.8 mM), suggesting that their growth depends more on intracellular nitrogen concentration than on extracellular nitrogen in the culture medium [45].

Regarding the effects of different sodium nitrate concentrations on ARA production, it was found that in BG11 with 123.54 mg N/L $NaNO_3$ provided the highest ARA yield at 73.62 ± 5.84 mg/g of DCW, with a production rate of 4.26 ± 0.58 mg/L/day and ARA content of 4.28 ± 0.09 % of TFA, after 9 days of cultivation. It was followed by 61.77 mg N/L $NaNO_3$, which gave an ARA production at 47.44 ± 4.89 mg/g of DCW and a production rate of 3.77 ± 1.15 mg/L/day with ARA content of 4.01 ± 0.03 % of TFA. However, increasing the concentration of $NaNO_3$ above 123.54 mg N/L resulted in a decrease in ARA production (**Tables 3** and **4**).

Table 3 Biomass and Production of Arachidonic acid in *Diplosphaera* sp. DMKU4.11.2 cultivating under various conditions.

Factors	Arachidonic acid (C20:4)			
	Biomass (g/L)	**Biomass production rate (g/L/day)**	**Production (mg/g of DCW)**	**Production rate (mg/L/day)**
Nitrogen sources[A]				
Sodium nitrate	0.29^{b} ± 0.03	0.02^{b} ± 0.00	95.70^{b} ± 7.25	2.73^{c} ± 0.08
Ammonium chloride	0.22^{b} ± 0.02	0.02^{b} ± 0.00	87.05^{b} ± 8.40	1.91^{b} ± 0.03
Urea	0.01^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00
Nitrogen levels[B] (mg N/l)				
0.00 (0 g/L)	0.10^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00
61.77 (0.38 g/L)	0.71^{b} ± 0.14	0.08^{c} ± 0.02	47.44^{b} ± 4.89	3.77^{b} ± 1.15
123.54 (0.75 g/L)	0.53^{b} ± 0.11	0.06^{c} ± 0.01	73.62^{c} ± 5.84	4.26^{b} ± 0.58
185.31 (1.13 g/L)	0.27^{a} ± 0.02	0.03^{b} ± 0.00	38.65^{ab} ± 3.11	1.14^{a} ± 0.01
247.07 (1.50 g/L)	0.21^{a} ± 0.00	0.02^{ab} ± 0.00	31.07^{a} ± 1.52	0.73^{a} ± 0.04
308.84 (1.88 g/L)	0.15^{a} ± 0.02	0.01^{ab} ± 0.00	46.43^{b} ± 3.95	0.76^{a} ± 0.20
Phosphorus ratios[B] (K_2HPO_4:KH_2PO_4)				
100:0	0.37^{a} ± 0.01	0.03^{a} ± 0.00	60.33^{b} ± 1.56	1.98^{a} ± 0.01
75:25	0.37^{a} ± 0.01	0.03^{a} ± 0.00	31.32^{a} ± 0.64	0.98^{a} ± 0.10
50:50	0.38^{a} ± 0.05	0.03^{a} ± 0.00	107.10^{c} ± 5.49	4.40^{b} ± 0.76
25:75	0.33^{a} ± 0.01	0.03^{a} ± 0.00	56.99^{b} ± 5.24	1.78^{a} ± 0.32
0:100	0.37^{a} ± 0.03	0.03^{a} ± 0.00	56.86^{b} ± 1.05	1.86^{a} ± 0.22
Phosphorus levels[C] (mg P/l)				

Factors	Arachidonic acid (C20:4)			
	Biomass (g/L)	**Biomass production rate (g/L/day)**	**Production (mg/g of DCW)**	**Production rate (mg/L/day)**
0.00	$0.12^{a} \pm 0.01$	$0.01^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
1.75	$0.26^{d} \pm 0.00$	$0.04^{d} \pm 0.00$	$51.41^{c} \pm 5.96$	$1.48^{bc} \pm 0.47$
3.50	$0.20^{b} \pm 0.01$	$0.03^{b} \pm 0.00$	$115.31^{e} \pm 2.03$	$3.23^{e} \pm 0.12$
5.25	$0.27^{d} \pm 0.01$	$0.04^{d} \pm 0.00$	$39.03^{b} \pm 4.95$	$1.33^{b} \pm 0.09$
7.00	$0.25^{cd} \pm 0.00$	$0.04^{d} \pm 0.00$	$53.30^{cd} \pm 1.58$	$1.98^{cd} \pm 0.09$
8.75	$0.23^{c} \pm 0.01$	$0.03^{c} \pm 0.00$	$60.54^{d} \pm 2.65$	$2.40^{d} \pm 0.27$
Sodium chloride[A] (mM)				
0	$0.54^{d} \pm 0.00$	$0.05^{d} \pm 0.00$	$45.64^{b} \pm 1.15$	$2.20^{b} \pm 0.06$
50	$0.36^{c} \pm 0.00$	$0.03^{c} \pm 0.00$	$93.51^{c} \pm 6.41$	$3.13^{b} \pm 0.28$
100	$0.52^{d} \pm 0.06$	$0.05^{d} \pm 0.01$	$88.39^{c} \pm 16.12$	$4.38^{c} \pm 1.29$
150	$0.23^{b} \pm 0.02$	$0.02^{b} \pm 0.00$	$74.66^{c} \pm 19.45$	$1.65^{ab} \pm 0.41$
200	$0.27^{b} \pm 0.03$	$0.02^{b} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
400	$0.13^{a} \pm 0.00$	$0.01^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$

Optimal yields of ARA across different experimental factors, with maximum yields achieved on varying cultivation days. Superscripts A, B and C correspond to cultivation periods of 10, 9 and 6 days, respectively. Values represent the mean ± standard deviation. Different superscripts within each column indicate statistically significant differences ($p < 0.05$) among conditions, as determined by Duncan's multiple range test. Abbreviations: DCW, dry cell weight; TFAs, total fatty acids.

BG11 medium containing 123.54 mg N/L $NaNO_3$ showed statistically significant differences in ARA production compared to other concentrations of sodium nitrate ($p < 0.05$) (**Table 3**, **Figure 3(D)**). Nitrogen concentration directly influences macromolecules in microalgal cells [46-48], with microalgae synthesizing lipids more efficiently to store energy under nitrogen-deficient conditions. The results of this study demonstrated that lipid concentration increases with the decreasing sodium nitrate concentration. These findings are consistent with previous research, which showed that under nitrogen-limited conditions, *Porphyridium purpureum* exhibited a higher ARA content compared to nitrogen-replete conditions. The study suggests that nitrogen limitation not only promotes fatty acid accumulation but also upregulates key genes and enzymes involved in ARA biosynthesis [49,50]. Furthermore, the results showed that the BG11 medium containing 123.54 mg N/L $NaNO_3$ yielded 2.52 times higher biomass concentration, 2.36 times higher ARA production and 2.69 times higher ARA content than standard BG11 medium containing 247.07 mg N/L $NaNO_3$ at 9 days of cultivation. This underscores the impact of nitrogen concentration on the growth and ARA production of *Diplosphaera* sp. DMKU4.11.2.

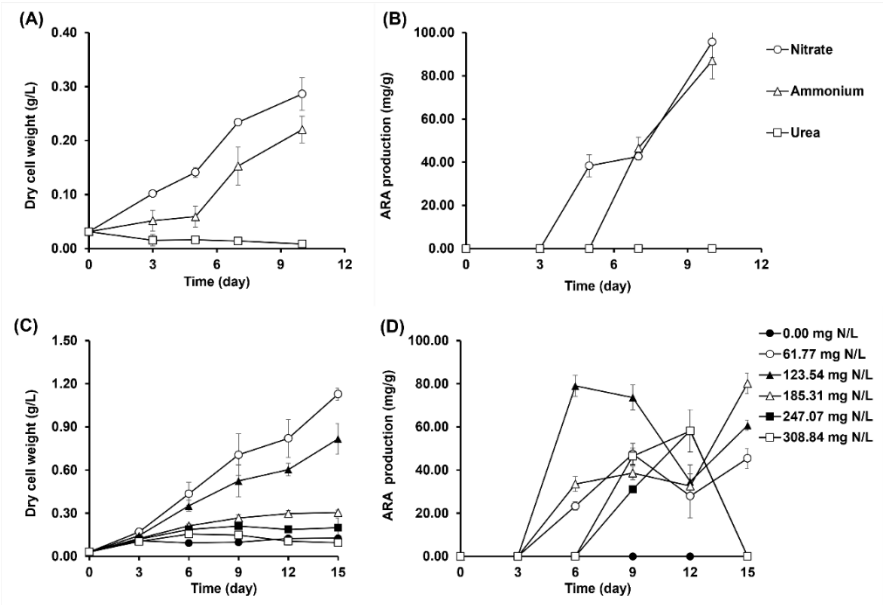

Figure 3 Effects of nitrogen sources and levels on growth and arachidonic acid production in *Diplosphaera* sp. DMKU4.11.2 after 15 days of cultivation. (A) Growth and (B) arachidonic acid production in different nitrogen sources. (C) Growth and (D) arachidonic acid production in different nitrogen levels; data are means ± standard deviation of 3 replicate cultures per variant.

Effects of phosphorus ratios on growth and ARA production

Phosphorus is an essential nutrient for growth and metabolism, and it is widely used as a buffer [51]. The effect of different phosphorus ratios in BG11 medium on biomass and ARA production in *Diplosphaera* sp. DMKU4.11.2 was investigated by the ratio of K_2HPO_4:KH_2PO_4. As a result, BG11 medium with K_2HPO_4:KH_2PO_4 ratio of 75:25 after 15 days of cultivation, showed the highest biomass concentration at 0.57 ± 0.01 g/L, followed by a ratio of 50:50 with the biomass concentration at 0.55 ± 0.04 g/L. While the standard BG11 medium with only K_2HPO_4 at a phosphorus ratio of 100:0, the biomass production was 0.52 ± 0.02 g/L (**Figure 4(A)**). These results are consistent with the study by Choi *et al.* [52] on *Chlorella* sp. UFPS019, which reported that the addition of phosphate buffer can increase the concentration of carbohydrates and proteins in the biomass by up to 45 and 40 %, respectively. Furthermore, it was observed that *Diplospheara* sp. DMKU4.11.2 grown in modified BG11 medium with K_2HPO_4:KH_2PO_4 ratio of 50:50 exhibited the highest ARA production at 107.10 ± 5.49 mg/g of DCW with an ARA production rate of 4.40 ± 0.76 mg/L/day and ARA content of 4.52 ± 0.00 % of TFAs. For the standard BG11 medium consisting of only K_2HPO_4 at a ratio of 100:0, the ARA production was 60.33 ± 1.56 mg/g of DCW with an ARA production rate of 1.98 ± 0.01 mg/L/day and ARA content of 2.60 ± 0.20 % of TFAs. ARA production, ARA production rate and ARA content of *Diplosphaera* sp. DMKU4.11.2 cultured in modified BG11 medium with K_2HPO_4:KH_2PO_4 ratio of 50:50 were significantly different from other phosphorus ratios at the 95 % confidence level ($p < 0.05$) (**Table 3**, **Figure 4(B)**). The results demonstrate that the modified BG11 medium with K_2HPO_4:KH_2PO_4 ratio of 50:50 resulted in a 1.77-fold increase in ARA production, a 2.22-fold increase in ARA production rate and a 1.73-fold increase in ARA content compared to the standard BG11 medium. Thus, our findings confirm that lipid accumulation can indeed be stimulated under stress conditions.

Effects of phosphorus levels on growth and ARA production

To explore the optimal phosphorus level, the study investigated various phosphorus levels. It was observed that the biomass increased with rising phosphorus level. However, excessively high phosphorus levels resulted in growth inhibition. In the modified BG11 medium with an initial phosphorus level of 7.00 mg P/L, the highest biomass recorded was 0.78 ± 0.02 g/L at 15 days. This

was followed by another peak of 0.75 ± 0.03 g/L observed at 5.25 mg P/L**.** However, when the phosphorus level was further increased to 8.75 mg P/L, the biomass decreased to 0.68 ± 0.03 g/L (**Figure 4(C)**). Consistent with the research of Choi *et al*. [52], culturing *Haematococcus pluvialis* NIES-144 in medium with the phosphate buffer concentration increased from 0.1 to 0.5 and 1.0 mM resulted in a reduction in biomass of approximately 60 and 43 %, respectively. Furthermore, the modified BG11 medium with 3.50 mg P/L phosphorus produced the highest ARA at 115.31 ± 2.04 mg/g of DCW with a production rate of 3.23 ± 0.12 mg/L/day and ARA content of 5.05 ± 0.02 % of TFA at 6 days of cultivation (**Figure 4(D)**). Furthermore, it produced the highest γ-linolenic acid at 21.57 ± 0.76 % of TFAs. The results are consistent with the research of Su *et al.* [53], which demonstrated that phosphate limitation enhances enhance the production of unsaturated fatty acids and ARA in *Porphyridium purpureum*. The findings suggest that phosphate limitation may increase the activity of enzymes such as Δ6-desaturase, which converts C16:0 to C18:2, thereby increasing the selectivity for unsaturated fatty acids. Furthermore, this effect may stimulate ARA biosynthesis by activating the ω6 pathway. Statistical analysis revealed that ARA production, production rate and ARA content at phosphorus level of 3.50 mg P/L were significantly different from other phosphorus levels at the 95 % confidence level ($p < 0.05$) (**Tables 3** and **4**). Compared with the Standard BG11 medium, which produced ARA 95.70 ± 7.25 mg/g of DCW and ARA content 2.78 ± 0.32 % of TFAs after 10 days of culture, the results showed that Modified BG11 with 3.50 mg P/L phosphorus produced ARA 1.20 times higher and ARA content 1.81 times higher. Moreover, the ARA production time was shorter. The difference of Standard BG11 medium and Modified BG11 medium in this research is that Standard BG11 contains $NaNO_3$ 1.500 g/L and K_2HPO_4 0.040 g/L, while Modified BG 11 contains $NaNO_3$ 0.750 g/L, K_2HPO_4 0.010 g/L and KH_2PO_4 0.008 g/L (**Table S1**). The results of this study demonstrate that cultivating the microalga *Diplosphaera* sp. DMKU4.11.2 in BG11 medium with optimal potassium phosphate level leads to improve growth and ARA production compared to the standard BG11 medium, which contains only 1 source of phosphorus, dipotassium hydrogen phosphate.

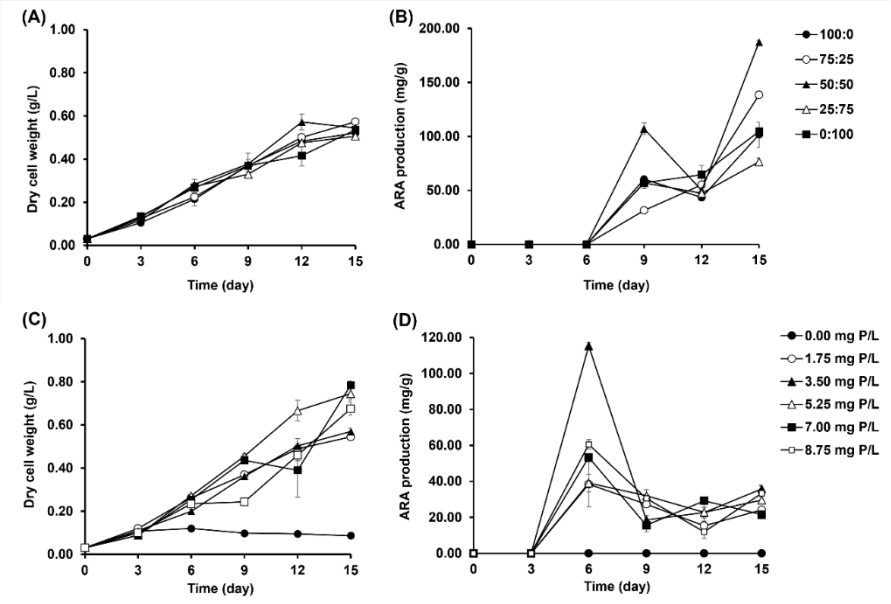

Figure 4 Effects of phosphorus ratios and levels on growth and arachidonic acid production in *Diplosphaera* sp. DMKU4.11.2 after 15 days of cultivation. (A) Growth and (B) arachidonic acid production in different phosphorus ratios. (C) Growth and (D) arachidonic acid production in different phosphorus levels; data are means ± standard deviation of 3 replicate cultures per variant.

Understanding how nitrogen and phosphorus levels affect both biomass and ARA production is crucial for designing sustainable microalgal cultivation systems. Optimizing nutrient levels to maximize ARA production, rather than just focusing on biomass, is essential. If lower nitrogen and phosphorus levels are needed for better ARA yields, adjusting these nutrient inputs can help reduce costs. Furthermore, sourcing nitrogen and phosphorus from waste streams, such as agricultural runoff or wastewater, can recycle waste into valuable products like biofuels, aquaculture feed or other bio-based materials [54,55]. This approach not only reduces the environmental impact of microalgae production but also enhances the sustainability of the system.

Effects of sodium chloride on growth and ARA production

Investigation of the effects of NaCl on growth and ARA production in *Diplosphaera* sp. DMKU4.11.2 was performed in 6 different concentrations of sodium chloride, namely 0, 50, 100, 150, 200 and 400 mM. It was observed that BG11 medium without NaCl at 0 mM showed the highest biomass concentration at 0.84 ± 0.00 g/L after 15 days of cultivation, followed by 50 and 100 mM NaCl with biomass of 0.68 ± 0.05 and 0.48 ± 0.08 g/L, respectively. The lowest biomass concentration of 0.15 ± 0.00 g/L was observed in the medium containing 400 mM NaCl. When cultured for 10 days in media containing 0 and 50 mM NaCl, the biomass was 0.54 ± 0.00 and 0.36 ± 0.00 g/L (**Figure 5(A)**). The results indicated that increasing NaCl concentrations resulted in decreased growth of the microalga *Diplosphaera* sp. DMKU4.11.2, and it could grow in modified BG11 medium containing up to 150 mM NaCl, as evidenced by the increase in biomass concentrations. Since *Diplosphaera* sp. DMKU4.11.2 is a freshwater microalga, so it can grow in low salinity. At NaCl concentrations of 100 mM and above, the biomass gradually decreased after 10 days. This decline may be attributed to microalgae being cultivated under prolonged stress conditions, ultimately leading to growth inhibition. This finding aligns with numerous reports of salt stress inhibiting growth in various photosynthetic organisms [56-58]. Pancha *et al*. [59] reported reduced biomass in the microalga *Scenedesmus* sp. CCNM 1077 when sodium chloride was introduced to the culture medium**.** Microalga *Diplosphaera* sp. DMKU4.11.2 produced the highest ARA yield of 93.51 ± 6.41 mg/g of DCW, with the ARA production rate of 3.13 ± 0.28 mg/L/day with a maximum ARA content of 9.57 ± 1.67 % of TFAs and γ-linolenic acid at 20.26 ± 0.68 % of TFAs in BG11 medium supplemented with 50 mM NaCl after 10 days of cultivation. At a NaCl concentration of 100 mM, the ARA production was 88.39 ± 16.12 mg/g of DCW, with ARA production rate of 4.38 ± 1.29 mg/L/day and ARA content 5.86 ± 0.74 % of TFAs. The highest ARA content at a NaCl concentration of 50 mM was significantly different from other NaCl concentrations ($p < 0.05$) (**Tables 3** and **4, Figure 5(B)**). These results suggest that the addition of salt to the medium enhances ARA production compared to a normal medium. This observation is consistent with the findings of Sinetova *et al.* [60], who reported that salinity can alter the fatty acid profile, favoring the accumulation of certain polyunsaturated fatty acids. In particular, ARA may be selectively preserved in polar lipids due to its vital role in the stress response. From this experiment, *Diplosphaera* sp. DMKU4.11.2 cultured for 10 days in standard BG11 media had the highest biomass production of 0.29 ± 0.03 g/L with growth rate 0.02 ± 0.00 g/L/day, ARA production of 95.70 ± 7.25 mg/g of DCW, with ARA production rate of 2.73 ± 0.08 mg/L/day and ARA content of 2.42 ± 0.33 % of TFAs. While in modified BG11 medium containing 50 mM NaCl, the biomass was 0.36 ± 0.00 g/L and growth rate 0.03 ± 0.00 g/L/day, ARA production of 93.51 ± 6.41 mg/g of DCW, with ARA production rate of 3.13 ± 0.28 mg/L/day and ARA content of 9.57 ± 1.67 % of TFAs. Therefore, the modified BG11 medium containing 50 mM NaCl increased growth 1.24-fold, ARA content 3.44-fold, ARA production rate 1.14-fold.

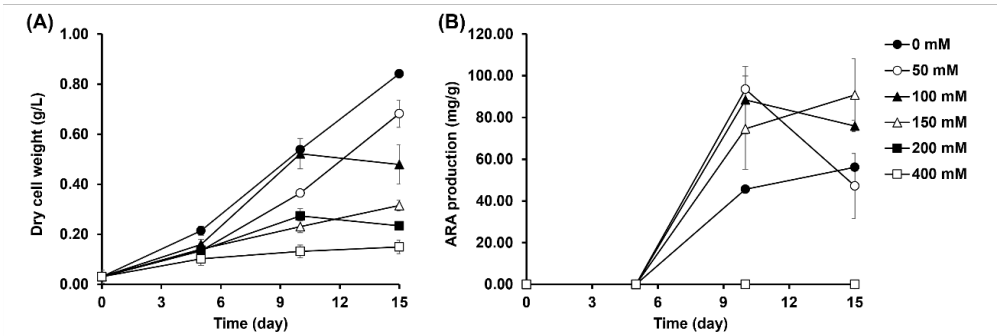

Figure 5 Effects of sodium chloride on growth and arachidonic acid production in *Diplosphaera* sp. DMKU4.11.2 after 15 days of cultivation. (A) Growth and (B) arachidonic acid production; data are means ± standard deviation of 3 replicate cultures per variant.

Table 4 Comparison of fatty acid composition of *Diplosphaera* sp. DMKU4.11.2 under different culture conditions.

Factor		Fatty acids content (% of TFA)						
		14:0	**16:0**	**18:0**	**18:1**	**18:2**	**γ-18:3(n-6)**	**20:4(n-6)**
Media	AF6	$3.26^{bc} \pm 0.04$	$41.33^{b} \pm 0.57$	$0.00^{a} \pm 0.00$	$6.57^{a} \pm 0.11$	$23.02^{bc} \pm 0.75$	$22.63^{ab} \pm 0.48$	$2.13^{bc} \pm 0.03$
	BBM	$2.98^{ab} \pm 0.06$	$37.12^{b} \pm 1.21$	$1.28^{c} \pm 0.08$	$5.70^{a} \pm 0.27$	$20.99^{ab} \pm 0.50$	$26.32^{abc} \pm 0.73$	$2.57^{cd} \pm 0.06$
	BG11	$2.38^{a} \pm 0.39$	$39.31^{b} \pm 0.51$	$0.00^{a} \pm 0.00$	$6.73^{a} \pm 0.06$	$21.84^{bc} \pm 0.50$	$27.40^{bc} \pm 1.29$	$2.78^{d} \pm 0.32$
	Modified Chu 13	$4.01^{cd} \pm 0.12$	$42.16^{b} \pm 2.45$	$2.25^{d} \pm 0.06$	$8.78^{b} \pm 0.59$	$18.71^{a} \pm 0.65$	$18.03^{a} \pm 2.10$	$0.92^{a} \pm 0.01$
	C	$3.06^{ab} \pm 0.16$	$35.07^{ab} \pm 4.33$	$1.02^{b} \pm 0.16$	$8.57^{b} \pm 0.45$	$20.21^{ab} \pm 1.07$	$25.15^{abc} \pm 0.28$	$1.89^{b} \pm 0.22$
	NSIII	$4.17^{d} \pm 0.64$	$28.53^{a} \pm 5.18$	$0.00^{a} \pm 0.00$	$7.09^{ab} \pm 1.53$	$24.53^{c} \pm 2.41$	$31.77^{c} \pm 7.70$	$2.78^{d} \pm 1.63$
Nitrogen sources	Sodium nitrate	$1.34^{a} \pm 0.01$	$41.82^{b} \pm 0.92$	$2.48^{b} \pm 0.48$	$15.68^{a} \pm 0.41$	$9.31^{b} \pm 0.28$	$8.22^{b} \pm 0.29$	$2.42^{b} \pm 0.33$
	Ammonium chloride	$2.20^{a} \pm 0.14$	$32.98^{a} \pm 2.16$	$2.85^{b} \pm 0.52$	$21.12^{b} \pm 0.24$	$15.59^{c} \pm 0.50$	$8.72^{b} \pm 1.30$	$1.90^{b} \pm 0.18$
	Urea	$13.64^{b} \pm 2.77$	$40.89^{b} \pm 0.87$	$0.00^{a} \pm 0.00$	$26.00^{c} \pm 2.19$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
Nitrogen Levels (mg N/L); $NaNO_3$ (g/L)	0.00; (0.00 g/L)	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	61.77; (0.38 g/L)	$2.42^{c} \pm 0.08$	$61.29^{e} \pm 1.15$	$1.35^{b} \pm 0.15$	$8.42^{b} \pm 4.41$	$10.66^{b} \pm 0.86$	$13.75^{c} \pm 5.37$	$4.01^{c} \pm 0.03$
	123.54; (0.75 g/L)	$1.34^{b} \pm 0.04$	$55.02^{de} \pm 0.86$	$1.77^{b} \pm 0.26$	$18.99^{c} \pm 4.03$	$12.69^{bc} \pm 0.30$	$8.59^{bc} \pm 2.55$	$4.28^{c} \pm 0.09$
	185.31; (1.13 g/L)	$1.48^{b} \pm 0.36$	$52.66^{cd} \pm 5.02$	$2.54^{c} \pm 0.30$	$22.79^{cd} \pm 2.20$	$11.91^{bc} \pm 1.84$	$7.01^{b} \pm 1.17$	$1.92^{b} \pm 0.16$
	247.07; (1.50 g/L)	$1.68^{b} \pm 0.17$	$46.72^{bc} \pm 4.90$	$2.92^{c} \pm 0.08$	$25.58^{cd} \pm 1.56$	$13.08^{bc} \pm 1.33$	$7.73^{bc} \pm 1.68$	$1.59^{a} \pm 0.14$
	308.84; (1.88 g/L)	$2.19^{c} \pm 0.24$	$42.23^{b} \pm 3.20$	$3.60^{d} \pm 0.18$	$27.83^{d} \pm 2.28$	$13.38^{c} \pm 0.62$	$7.59^{bc} \pm 0.46$	$1.47^{a} \pm 0.04$
Phosphorus ratios (K_2HPO_4: KH_2PO_4)	100:0	$1.36^{c} \pm 0.05$	$42.10^{b} \pm 0.45$	$1.09^{b} \pm 0.10$	$13.30^{a} \pm 0.23$	$14.40^{bc} \pm 0.64$	$14.75^{b} \pm 156$	$2.60^{b} \pm 0.20$
	75:25	$1.34^{c} \pm 0.06$	$48.82^{c} \pm 1.75$	$1.22^{b} \pm 0.01$	$17.27^{b} \pm 0.28$	$12.09^{a} \pm 0.19$	$12.66^{ab} \pm 0.14$	$1.69^{a} \pm 0.01$
	50:50	$1.39^{c} \pm 0.05$	$35.95^{a} \pm 2.61$	$1.20^{b} \pm 0.06$	$16.75^{b} \pm 0.23$	$15.50^{c} \pm 0.16$	$16.22^{b} \pm 3.36$	$4.52^{c} \pm 0.00$
	25:75	$1.09^{b} \pm 0.14$	$42.46^{b} \pm 2.57$	$1.71^{c} \pm 0.12$	$17.28^{b} \pm 0.67$	$13.99^{b} \pm 0.81$	$10.00^{a} \pm 1.06$	$2.72^{b} \pm 0.07$
	0:100	$0.00^{a} \pm 0.00$	$43.06^{b} \pm 0.16$	$0.00^{a} \pm 0.00$	$12.60^{a} \pm 0.82$	$13.20^{ab} \pm 0.39$	$14.99^{b} \pm 0.28$	$2.63^{b} \pm 0.17$
Phosphorus levels (mg P/L)	0.00	$1.84^{ab} \pm 0.26$	$37.12a \pm 5.65$	$2.11^{b} \pm 0.25$	$17.06^{d} \pm 3.63$	$19.09^{b} \pm 3.26$	$10.37^{a} \pm 3.30$	$0.00^{a} \pm 0.00$
	1.75	$2.61^{b} \pm 0.69$	$65.43^{b} \pm 6.34$	$1.60^{ab} \pm 0.19$	$7.47^{bc} \pm 1.56$	$11.64^{a} \pm 0.31$	$17.20^{b} \pm 0.76$	$2.80^{b} \pm 0.05$
	3.50	$1.45^{a} \pm 0.05$	$26.85^{a} \pm 1.05$	$1.15^{a} \pm 0.12$	$6.41^{ab} \pm 0.60$	$8.99^{a} \pm 0.08$	$21.57^{b} \pm 0.76$	$5.05^{c} \pm 0.02$
	5.25	$2.19^{ab} \pm 0.17$	$34.40^{a} \pm 7.98$	$1.29^{a} \pm 0.19$	$12.01^{c} \pm 2.18$	$13.35^{a} \pm 2.91$	$18.58^{b} \pm 5.25$	$2.77^{b} \pm 0.02$
	7.00	$1.76^{a} \pm 0.02$	$37.95^{a} \pm 0.01$	$1.52^{ab} \pm 0.49$	$3.21^{ab} \pm 0.05$	$9.08^{a} \pm 1.09$	$16.45^{ab} \pm 0.41$	$3.45^{c} \pm 0.25$
	8.75	$1.66^{a} \pm 0.22$	$34.71^{a} \pm 0.21$	$1.07^{a} \pm 0.12$	$2.48^{a} \pm 0.40$	$8.68^{a} \pm 0.26$	$20.93^{b} \pm 1.08$	$3.74^{d} \pm 0.00$

Factor		Fatty acids content (% of TFA)						
		14:0	**16:0**	**18:0**	**18:1**	**18:2**	**γ-18:3(n-6)**	**20:4(n-6)**
Sodium chloride levels (mM)	0	$8.23^{b} \pm 0.08$	$41.63^{b} \pm 0.15$	$0.00^{a} \pm 0.00$	$4.77^{a} \pm 0.11$	$7.52^{a} \pm 0.58$	$16.28^{ab} \pm 0.09$	$4.73^{b} \pm 0.09$
	50	$8.99^{b} \pm 1.90$	$37.21^{b} \pm 5.38$	$0.00^{a} \pm 0.00$	$7.85^{a} \pm 1.82$	$11.34^{a} \pm 0.00$	$20.26^{b} \pm 0.68$	$9.57^{c} \pm 1.67$
	100	$3.96^{a} \pm 1.35$	$26.88^{a} \pm 6.65$	$0.00^{a} \pm 0.00$	$5.97^{a} \pm 3.49$	$10.29^{a} \pm 5.49$	$16.86^{ab} \pm 9.29$	$5.86^{b} \pm 0.74$
	150	$5.39^{a} \pm 0.27$	$40.87^{b} \pm 4.12$	$0.00^{a} \pm 0.00$	$8.29^{a} \pm 0.37$	$13.21^{a} \pm 0.57$	$13.45^{ab} \pm 0.27$	$4.79^{b} \pm 0.07$
	200	$3.66^{a} \pm 0.37$	$42.85^{b} \pm 2.84$	$0.00^{a} \pm 0.00$	$21.51^{b} \pm 0.24$	$12.49^{a} \pm 0.08$	$7.54^{a} \pm 0.19$	$0.00^{a} \pm 0.00$
	400	$8.69^{b} \pm 1.52$	$34.93^{ab} \pm 0.13$	$0.00^{a} \pm 0.00$	$26.13^{c} \pm 1.67$	$11.76^{a} \pm 0.37$	$7.58^{a} \pm 0.06$	$0.00^{a} \pm 0.00$

TFAs: Total fatty acids. Each value represents the mean ± standard deviation. In each column, different letters above paired values indicate that the values obtained from analysis of variance are significantly different ($p < 0.05$).

There are few reports of ARA production in microalgae, mostly found in marine algae such as *Porphyridium cruentum*, *Porphyridium purpureum*, *Nannochloropsis oculate* and *Koliella antarctica* [14,39,53,61,62]. In freshwater algae, it was found in *Parietochloris incisa* [63-66]. This research is a new report on the study of the optimum cultivation conditions for ARA production in the freshwater microalga *Diplosphaera* sp. DMKU4.11.2 isolated from Thailand (**Table 5**). Moreover, *Diplosphaera* sp. DMKU4.11.2 isolated from Thailand cultured in BG11 medium containing 3.50 mg P/L phosphorus produced ARA at 115.31 ± 2.03 mg/g of DCW after 6 days of culture, while medium containing 50 mM NaCl produced ARA at 93.51 ± 6.41 mg/g of DCW after 10 days of culture. The results indicated that *Diplosphaera* sp. DMKU4.11.2 could produce higher ARA than *Porphyridium purpureum* [14], *Koliella antarctica* [62], *Lobosphaera incisa* (*Parietochloris incisa*) [65], and the culture period was much shorter. Future optimization studies could explore alternative stress factors, such as varying light intensities, temperature, or combined nutrient modifications, to further enhance ARA production. Investigating metabolic pathways in detail under these conditions may also provide insights into regulatory mechanisms driving ARA production. Furthermore, linking these findings to industrial applications could position *Diplosphaera* sp. DMKU4.11.2 as a promising candidate for sustainable and cost-effective ARA production. For instance, the use of low-cost BG11 medium and shorter cultivation periods could reduce production costs, making this strain suitable for large-scale aquaculture or nutraceutical industries. Therefore, if further research is conducted, *Diplosphaera* sp. DMKU4.11.2 may be used as an alternative ARA source for industrial applications.

Table 5 Comparison of arachidonic acid content in *Diplosphaera sp.* DMKU4.11.2 with other microalgae.

Microalgae	Culture medium	Temp (°C)	Light intensity (μmol/m²/s), photo period (Light: Dark h)	Nutrient status	Cultivation time (day)	Biomass yield	ARA production	References
Porphyridium cruentum[a]	Jones' medium	30	30	-	3	3 mg/L	42.1 % of TFAs	[61]
Porphyridium purpureum[a]	ASW	25	110, 24:0 h	-	18	13.07 g/L	125.73 mg/L	[14]
	ASW	25	165, 24:0 h	$NaHCO_3$ 0.8 g/L	18	11.47g/L	9.13 mg/g	[53]
	ASW	25	165, 24:0 h	$NaHCO_3$ 0.8 g/L, KH_2PO_4 0.01g/L	18	9.15 g/L	10.08 mg/g	
Nannochloropsis oculate[a]	Walne's medium	23	300, 12:12 h	$NaNO_3$ 1.18 mM	5	0.92 g/L	11.3 % of TFAs	[39]
Koliella antarctica[a]	BBM	15	500, 24:0 h	-	18	2.37 g/L/day 11.68 g/L	9.6 mg/g, 3.5 % of TFAs	[62]

Microalgae	Culture medium	Temp (°C)	Light intensity (µmol/m²/s), photo period (Light: Dark h)	Nutrient status	Cultivation time (day)	Biomass yield	ARA production	References
Parietochloris incisa[b]	BG11	25	115, 24:0 h	-	15 - 20	5.4 g/L	43 % of TFAs, 8.9 % of DCW	[63]
	BG11	25	200, 24:0 h	-	17	0.39 mg/L/day	47 % of TFAs	[64]
	BG11	26	250, 24:0 h	-	38	8.8 g/L	73 mg/g	[65]
Lobosphaera incisa[b] *(Parietochloris incisa)*	BG11	25	100	-	7	6 g/L	36.7 % of TFAs	[66]
Diplosphaera sp. DMKU4.11.2[b]	BG11	25	40, 24:0 h	$NaNO_3$ 0.75 g/L, Potassium phosphate (1:1),	6	0.20 g/L	115.31 mg/g, 3.23 mg/L/day	This study

a: Marine algae; *b*: Freshwater algae; Temp: Temperature; DCW: Dry cell weight; TFAs: Total fatty acids

Conclusions

The freshwater microalga *Diplosphaera* sp. DMKU4.11.2, isolated from a bryophyte in Thailand, demonstrated significant potential for ARA production under optimized cultivation conditions. The use of modified BG11 medium with adjusted sodium nitrate, phosphorus levels, along with the addition of 50 mM NaCl significantly enhanced ARA content and biomass production compared to the standard medium. This strain achieved higher ARA production rates within a shorter cultivation period compared to previously studied microalgae, highlighting its promise as an alternative, sustainable source for ARA. These findings support the potential of *Diplosphaera* sp. DMKU4.11.2 for industrial applications, particularly in nutraceuticals, aquafeeds and pharmaceuticals. Future research could focus on scaling up cultivation and exploring additional stress conditions to further optimize ARA yield.

Acknowledgements

This research was partially supported by the Graduate Program Scholarship from The Graduate School, Kasetsart University, Thailand.

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Supplementary Material

Table S1 The composition of standard BG 11 and modified BG 11 media.

Chemical component	Concentration (g/L)	
	Standard BG 11	**Modified BG 11**
$NaNO_3$	1.500	0.750
K_2HPO_4	0.040	0.010
KH_2PO_4	-	0.008
$MgSO_4 \cdot 7H_2O$	0.075	0.075
$CaCl_2 \cdot 2H_2O$	0.036	0.036
Na_2CO_3	0.020	0.020
Ferric ammonium citrate	0.006	0.006
Citric acid	0.006	0.006
EDTA	0.001	0.001
Trace metal A5	1 mL	1 mL
Trace metal A5		
H_3BO_3	2.860	
$MnCl_2.4H_2O$	1.810	
$ZnSO_4.7H_2O$	0.222	
$NaMoO_4.5H_2O$	0.039	
$CuSO_4.5H_2O$	0.079	
$Co(NO_3)_2.6H_2O$	0.049	
Distilled water	1000 mL	

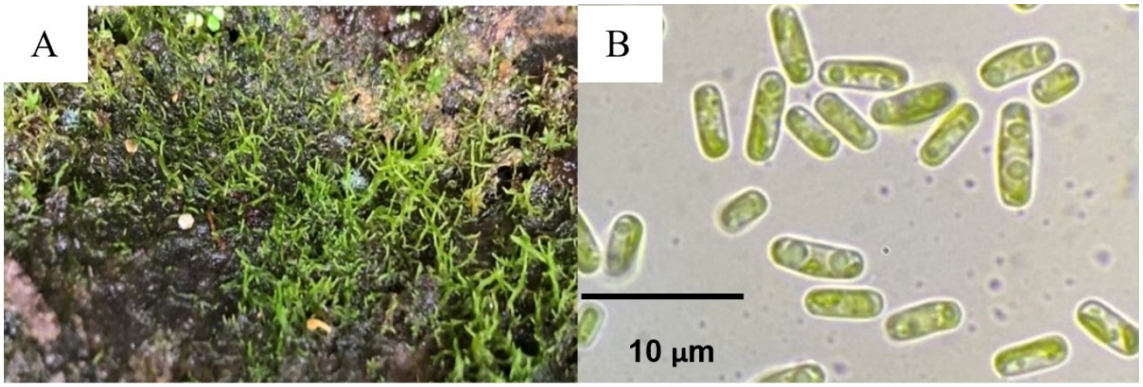

Figure S1 Bryophyte collected for microalgae isolation (A) Light microscope image (400X) of *Diplosphaera sp.* DMKU4.11.2 (B).