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Research Article

Synergistic Effects of Salinity and AMPEP Concentrations on the Growth, Pigment Accumulation, and Biochemical Composition of *Chlorella sorokiniana* Culture

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ABSTRACT

Microalgae, particularly *Chlorella sorokiniana*, are widely recognized for their applications in aquaculture, biofuel production, and nutraceutical industries due to their high biomass yield and valuable biochemical composition. However, optimizing culture conditions remains essential for improving growth and metabolite production. This study investigated the combined effects of sodium chloride (NaCl) and Ascophyllum (Acadian) Marine Plant Extract Powder (AMPEP) concentrations in BG-11 medium on the growth, pigment accumulation, and total phenolic content of *C. sorokiniana*. Eight treatments were evaluated: T1 (control BG-11 only), T2 (AMPEP only), T3 (NaCl only), T4 (NaCl + AMPEP), T5 (NaCl + AMPEP + BG-11), T6 (15 g L^{-1} NaCl + 100 mg L^{-1} AMPEP + BG-11), T7 (20 g L^{-1} NaCl + 150 mg L^{-1} AMPEP + BG-11), and T8 (25 g L^{-1} NaCl + 200 mg L^{-1} AMPEP + BG-11). Results showed that T6 achieved the highest cell density, reaching 10.14-fold cell mL^{-1} compared with the control. The highest specific growth rate and dry weight were observed in T8 at 0.18±0.01 day^{-1} and 0.14±0.00 g L^{-1}, respectively. Cell size was significantly larger ($p<0.05$) in T6, T7, and T8. For pigment accumulation, T5 recorded the highest chlorophyll a (25.54±1.42 μg mL^{-1}), total carotenoids (97±0.88 μg mL^{-1}), and while the same treatment also yielded the highest total phenolic content (8.72±0.87 mg GAE g^{-1} DW). These findings demonstrate that NaCl-AMPEP supplementation enhances biomass, pigment accumulation, and biochemical composition in *C. sorokiniana*.

1. Introduction

Microalgae are microscopic organisms found in marine and freshwater environments (Shahid *et al.* 2019; Neto & Pinto 2019). It is a unicellular, photosynthetic microorganism that harnesses sunlight to produce energy and organic compounds through photosynthesis (Tanvir *et al.* 2021; Khan *et al.* 2022). Microalgae are noteworthy microorganisms that play a vital role in aquaculture production, it serve as the primary source of food in the early life stage of many aquatic organisms (Brown & Blackburn 2013; Chowdhury & Das 2023) due to their rich nutrients, such as proteins, lipids, vitamins and minerals (Udayan *et al.* 2023) that essential for larval development (Chowdhury & Das 2023). In addition to their role as a food source, microalgae contribute significantly to bioremediation by absorbing excess nutrients such as nitrogen and phosphorus from aquaculture wastewater, thereby reducing environmental pollution and maintaining water quality (Markou & Georgakakis 2011; Mishra *et al.* 2022; Sarri *et al.* 2024c).

Propagation of microalgae is a crucial step in ensuring their availability for aquaculture and other applications (Han *et al.* 2019; Durmaz & Erbil 2020; Erbil *et al.* 2021, 2022). Microalgae like *Tetraselmis, Nannochloropsis, Chaetoceros,* and *Chlorella* are commonly used in aquaculture (Bhambri *et al.* 2023; Kumar *et al.* 2023;

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Sarri *et al.* 2024a, 2024b) their growth involves several techniques such as culture methods, nutrient enrichment, and physicochemical parameters (Rai & Rajashekhar 2014; Tandon & Jin 2017; Kumar & Thomas 2019; Sarri & Elp 2024). Moreover, microalgae of the genus *Chlorella* are among the species used in aquaculture because of their small cell size, which is easily digested by newly hatched aquatic organisms (Shield & Lupatsch 2013). Furthermore, *Chlorella* is rich in lipids, proteins, fatty acids, vitamins, minerals, and high pigment content, including chlorophyll a and b (Griffiths *et al.* 2012), as well as carotenoids (Guedes & Malcata 2012; Sarri *et al.* 2024c). Several studies have been explored, and various strategies have been used to enhance their growth and optimize biomass production in *Chlorella* species (Daliry *et al.* 2017; Cao *et al.* 2020; Sarri & Elp 2024).

Cultivation of *Chlorella* sp. requires nutrients to enhance biomass production, such as nitrogen, phosphorus, and potassium (Mtaki *et al.* 2021; Tavares *et al.* 2023; Sarri & Elp 2024). Various growth enhancers have been studied, among which BG-11 medium is one of the media used to enhance *Chlorella* sp. biomass production (Durmaz & Erbil 2020; Elakbawy *et al.* 2023; Pandey *et al.* 2023; Elp *et al.* 2024). Recently, Acadian Marine Plant Extract Powder (AMPEP) proved to be a good growth enhancer and pigment enhancer in the study of *Chlorella* sp. and *Nannochloropsis* sp. culture (Sarri *et al.* 2024a, 2024b). Additionally, Salt has been used as a nutrient essential to the growth of *Chlorella* sp. and has improved lipid and pigment content (Benavente-Valdés *et al.* 2016; Ali *et al.* 2021; Liu *et al.* 2021; Sarri & Elp 2024). However, few studies have examined the incorporation of different growth enhancers into the cultivation of *Chlorella* sp. Thus, this study investigates the synergistic effects of sodium chloride (NaCl) and AMPEP concentrations in a nutrient medium on the growth, pigment accumulation, and total phenolic content of *C. sorokiniana* culture.

2. Materials and Methods

2.1. Experimental Culture Condition of Microalgae

Microalga *C. sorokiniana* culture was conducted at the Multi-Species Hatchery Phycology Laboratory of the College of Fisheries, Mindanao State University Tawi-Tawi College of Technology and Oceanography, located in Sanga-Sanga, Bongao, Tawi-Tawi, Philippines (Figure 1). 500 mL of improvised long-neck glass bottles were used in *C. sorokiniana* culture. The BG-11 medium was utilized as a nutrient medium (Tables 1 and 2). The composition of Acadian Marine Plant Extract Powder (AMPEP) is shown in Table 3. For salinity concentration, different concentrations of sodium chloride (NaCl) were added to glass bottles containing a nutrient medium and AMPEP, as shown in Table 4. The experimental treatment was done in triplicate with a research design of Complete Randomized Design (CRD). Each solution was autoclaved for 20 minutes at 121°C. After autoclaving, experimental samples were inoculated

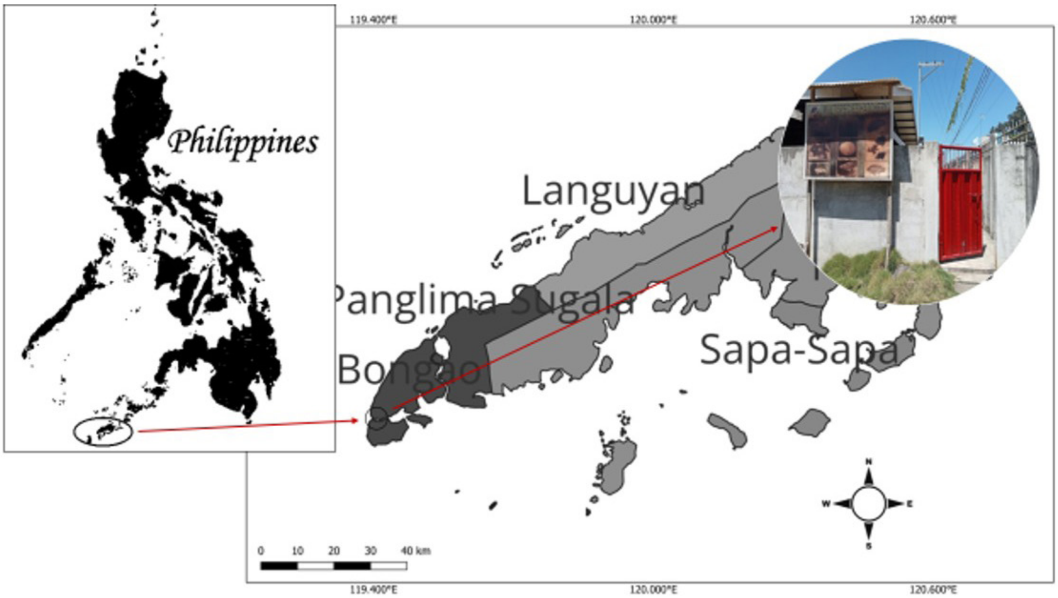

Figure 1. Map of the research site

Table 1. BG-11 nutrient medium (Erbil *et al.* 2021; Sarri *et al.* 2024a)

Solution A	For 500 mL
$NaNO_3$	75.0 g
Solution B	**For 500 mL**
K_2HPO_4	2.0 g
$MgSO_4{\bullet}7H_2O$	3.75 g
$CaCl_2{\bullet}2H_2O$	1.80 g
Citric acid	0.30 g
Ammonium ferric citrate green	0.30 g
$EDTANa_2$	0.05 g
Na_2CO_3	1.00 g

Table 2. Trace element composition

Trace element solution	For 1,000 mL
H_3BO_3	2.86 g
$MnCl_2{\bullet}4H_2O$	1.81 g
$ZnSO_4{\bullet}7H_2O$	0.22 g
$Na_2MoO_4{\bullet}2H_2O$	0.39 g
$CuSO_4{\bullet}5H_2O$	0.08 g
$Co(NO_3)2{\bullet}6H_2O$	0.05 g

Table 3. Composition of Acadian marine plant extract powder (AMPEP) 0.7 – 0.09 – 14.1 from *Ascophyllum nodosum* (The composition was obtained from the Acadian Seaplants, Product of Canada) (Sarri *et al.* 2024b)

Physical analysis	
Appearance	Brownish-black crystals
Odor	Marine odor
Solubility in water	100%
Typical analysis	
Minerals (Ash)	45-50%
Maximum moisture	6.5%
Minimum alginic acid	10%
Minimum Mannitol	4%
Minimum Amino acids	4%
Nitrogen (N) as organic	0.7%
Phosphorus (P) as water-soluble	0.09%
Total potassium (K)	14.1%

Table 4. Experimental treatments with salinity, AMPEP, and nutrient medium in Microalga *C. sorokiniana*

Experimental treatment	NaCl (g L^{-1})	AMPEP (mg L^{-1})	BG-11 medium (mL L^{-1})
T1 (Control)	0	0	10
T2	0	50	0
T3	10	0	0
T4	10	50	0
T5	10	50	10
T6	15	100	10
T7	20	150	10
T8	25	200	10

at an initial density of 4.56×10^5 cells mL^{-1}. Cultures were maintained under continuous illumination (24 h photoperiod) using fluorescent lamps (MASTER TL-D Super 80 36W/865 1SL/25). The culture temperature was maintained at 20±1°C using an air-conditioning system. Continuous aeration was supplied by an air pump, and sterile syringe filters (0.2 µm) were attached to the aeration lines to minimize contamination (Figure 2). In addition, we have explicitly stated that the experiment was conducted using a Completely Randomized Design (CRD) with eight treatments and three replicates per treatment.

2.2. Growth Response Analysis

Each experimental sample of *C. sorokiniana* microalgal cultures was collected for cell counting and analysis every 3 days. A Neubauer hemocytometer was used to count cells daily under a light microscope, and contamination was visually checked daily. An analysis of the biomass of microalgae was conducted on a dry-weight basis. The dried weight of microalgae was determined by drying 5 mL of each experimental sample in an oven at 105°C for 2 hours. The specific growth rate (µ) was calculated by the following (Sanuddin *et al.* 2023).

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1}$$

Where:
N_2: cell number at the time (t2)
N_1: beginning cell number at a time (t1)

2.3. Cell Size Measurement

Photographs of the cells were captured and analyzed using ImageJ software (National Institutes of Health, USA). Measurements were taken within the Neubauer chamber, and a total of 10 randomly selected cells were measured.

2.4. Pigment Analysis

Pigment analysis was conducted following the method of Durmaz & Erbil (2020). The chlorophyll a and total carotenoid levels of *C. sorokiniana* were determined using spectrophotometric analysis. In the experimental culture, a 5 mL test sample was centrifuged at 3,500 rpm for 10 minutes, after which the supernatant was discarded. The remaining sample was resuspended

Figure 2. Experimental set-up. The glass bottles were enriched with different salinity concentrations (g L^{-1}) and AMPEP concentration in a nutrient medium

in 5 mL of methanol and vortexed for 30 seconds to ensure homogenization. The samples were then mixed again and centrifuged under the same conditions. The supernatant was analyzed using spectrophotometry, with absorbance readings taken at 666 nm for chlorophyll a and 475 nm for total carotenoids, using the formulas provided below for final calculations.

Chlorophyll a (µg/ml) = 13.9 A_{666} (Macıas-Sánchez *et al.* 2005)
A_{666} wavelength 666 nm absorbance value

Total carotenoids (µg/ml) = 4.5 A_{475} (Zou & Richmond 2000)
A_{475} wavelength 475 nm absorbance value

2.5. Total Phenolic Content Analysis

The Total phenolic content (TPC) of microalgae of *C. sorokiniana* was analyzed using the Folin–Ciocalteu (FC) method with modifications (Singleton *et al.* 1999). Using a centrifuge, *C. sorokiniana* was extracted from the water at 3,500 rpm to obtain 5 mg of concentrated samples for each replicate. The 5 mg samples were placed in test tubes containing 1 mL of methanol. The samples were then placed in the beaker and heated to 50°C on a hot plate for 1 hour. Afterward, each of the samples was shaken with a vortex every 10 minutes. After 1 hour of heating, the samples were centrifuged at 3,500 for 5 minutes. 0.100 mL of extracted *C. sorokiniana* was placed in a separate test tube. For each extract sample, 1.500 mL of methanol, 0.100 mL of FC reagent, and 0.300 mL of baking soda were mixed by vortexing for 30 seconds. Again, the samples were placed inside the beaker, heated at 50°C using a hot plate for 2 hours, and each of the samples was shaken with a vortex every 10 minutes. Finally, the sample's TPC was analyzed using a spectrophotometer at 765 nm. The results of the analysis were reported as milligrams of gallic acid equivalents (GAE) per gram of dry biomass (mg GAE/g DW).

2.7. Statistical Analysis

The collected data on cell density, growth response, pigment accumulation, and TPC of the microalgal *C. sorokiniana* culture were analyzed using IBM SPSS Statistics version 21 at a significance level of $p<0.05$. Results were expressed as mean ± standard error of the mean (SEM). Statistical differences were determined using One-Way Analysis of Variance (ANOVA), while Levene's Test was performed to assess the homogeneity of variance. Duncan's Post-Hoc Test was applied to rank the means (Hairol *et al.* 2022; Sanuddin *et al.* 2023; Sarri *et al.* 2024c).

3. Results

3.1. Growth Response

Table 5 demonstrates the cell density of *Chlorella sorokiniana* cultured at varying concentrations of NaCl, AMPEP, and BG-11 medium. The initial cell density was set at 4.56×10^5 cell mL^{-1}, and the culture was conducted in triplicates. Based on the results of this study, after 24 days of cultivation, the cell density of T1, T2, T3, T4, T5, T6, T7, and T8 were $1.57\pm0.63 \times 10^6$ cell mL^{-1}, $2.70\pm0.57 \times 10^6$ cell mL^{-1}, $1.07\pm0.32 \times 10^6$ cell mL^{-1}, $1.93\pm0.24 \times 10^6$ cell mL^{-1}, $13.78\pm1.46 \times 10^6$ cell mL^{-1}, $15.92\pm2.04 \times 10^6$ cell mL^{-1}, $11.77\pm0.87 \times 10^6$ cell mL^{-1}, and $8.50\pm0.48 \times 10^6$ cell mL^{-1}, respectively. ANOVA revealed that T6 had significantly higher ($p<0.05$) cell density compared to T1. In addition, Table 6 shows the specific growth

Table 5. Cell Density (n × 10^6) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium

Treatment	Days of culture							
	DAY 3	DAY 6	DAY 9	DAY 12	DAY 15	DAY 18	DAY 21	DAY 24
T1 (10^6 cell mL^{-1})	4.47 ± 0.53^{a}	10.72 ± 0.24^{a}	12.98 ± 0.59^{a}	14.80 ± 2.33^{a}	12.08 ± 1.14^{b}	14.80 ± 2.33^{b}	14.03 ± 0.44^{b}	1.57 ± 0.63^{d}
T2 (10^6 cell mL^{-1})	1.93 ± 0.17^{b}	2.10 ± 0.19^{c}	2.50 ± 0.45^{c}	3.33 ± 0.46^{c}	2.68 ± 0.27^{c}	3.33 ± 0.46^{c}	2.63 ± 0.12^{c}	2.70 ± 0.57^{c}
T3 (10^6 cell mL^{-1})	0.55 ± 0.08^{c}	0.78 ± 0.17^{d}	0.92 ± 0.04^{d}	1.10 ± 0.03^{d}	1.32 ± 0.14^{d}	1.23 ± 0.19^{d}	1.30 ± 0.25^{d}	1.07 ± 0.32^{d}
T4 (10^6 cell mL^{-1})	1.10 ± 0.22^{c}	1.93 ± 0.39^{d}	1.83 ± 0.33^{d}	1.55 ± 0.05^{d}	1.42 ± 0.31^{d}	1.45 ± 0.06^{d}	1.47 ± 0.17^{d}	1.93 ± 0.24^{d}
T5 (10^6 cell mL^{-1})	0.93 ± 1.69^{c}	3.73 ± 1.17^{b}	12.03 ± 0.65^{a}	12.25 ± 0.58^{a}	22.00 ± 1.83^{a}	18.48 ± 2.49^{a}	14.53 ± 1.72^{b}	13.78 ± 1.46^{a}
T6 (10^6 cell mL^{-1})	0.43 ± 0.19^{c}	0.98 ± 0.04^{d}	6.98 ± 0.62^{b}	8.72 ± 1.04^{b}	12.73 ± 1.38^{b}	10.47 ± 0.79^{b}	16.17 ± 0.55^{a}	15.92 ± 2.14^{a}
T7 (10^6 cell mL^{-1})	0.23 ± 0.04^{c}	0.63 ± 0.19^{d}	1.47 ± 0.16^{d}	4.47 ± 0.07^{c}	4.28 ± 0.56^{c}	5.12 ± 0.47^{c}	7.27 ± 0.59^{c}	11.77 ± 0.87^{a}
T8 (10^6 cell mL^{-1})	0.18 ± 0.07^{c}	0.30 ± 0.05^{d}	0.67 ± 0.07^{d}	2.48 ± 0.12^{c}	2.38 ± 0.38^{c}	3.18 ± 0.56^{c}	4.97 ± 0.71^{c}	8.50 ± 0.48^{b}

Values are in SEM (standard error mean). T1 (0 g L^{-1} NaCl: 0 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T2 (0 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T3 (10 g L^{-1} NaCl: 0 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T4 (10 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T5 (10 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T6 (15 g L^{-1} NaCl: 100 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T7 (20 g L^{-1} NaCl: 150 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T8 (25 g L^{-1} NaCl: 200 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11). Column with the same letter are not significantly different ($p>0.05$)

Table 6. Specific growth rate (SGR, day^{-1}) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium

Treatment	Days of culture							
	DAY 3	DAY 6	DAY 9	DAY 12	DAY 15	DAY 18	DAY 21	DAY 24
T1 (day^{-1})	0.04±0.76	0.02±0.15	0.00±0.00	0.01±0.01	0.01±0.01	0.01±0.00	0.00±0.01	0.00±0.00
T2 (day^{-1})	0.03±0.48	0.00±0.00	0.01±0.02	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.00±0.00
T3 (day^{-1})	0.04±0.06	0.06±0.05	0.03±0.02	0.02±0.00	0.01±0.01	0.00±0.01	0.00±0.00	0.01±0.01
T4 (day^{-1})	0.07±0.28	0.01±0.00	0.03±0.01	0.02±0.01	0.01±0.01	0.00±0.01	0.00±0.00	0.01±0.01
T5 (day^{-1})	0.07± 0.23	0.07±0.22	0.04±0.14	0.00±0.00	0.04±0.00	0.01±0.00	0.01±0.00	0.00±0.00
T6 (day^{-1})	0.16± 0.09	0.09±0.17	0.01±0.00	0.02±0.01	0.03±0.01	0.01±0.01	0.02±0.00	0.00±0.0
T7 (day^{-1})	0.07± 0.24	0.03±0.17	0.03±0.01	0.09±0.01	0.00±0.01	0.01±0.00	0.02±0.00	0.02±0.01
T8 (day^{-1})	0.18±0.38	0.11±0.12	0.02±0.01	0.09±0.00	0.00±0.01	0.02±0.00	0.02±0.00	0.02±0.00

Values are in SEM (standard error mean). T1 (0 g L^{-1} NaCl: 0 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T2 (0 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T3 (10 g L^{-1} NaCl: 0 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T4 (10 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 0 mL L^{-1} BG-11); T5 (10 g L^{-1} NaCl: 50 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T6 (15 g L^{-1} NaCl: 100 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T7 (20 g L^{-1} NaCl: 150 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11); T8 (25 g L^{-1} NaCl: 200 mg L^{-1} AMPEP: 10 mL L^{-1} BG-11). Column with the same letter are not significantly different ($p>0.05$)

rate (SGR) of *C. sorokiniana* cultured under different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results of the study, the maximum SGR was achieved in T8 (0.18±0.01 day^{-1}), followed by T6 (0.16±0.02 day^{-1}), T4 (0.07±0.01 day^{-1}), T5 (0.07±0.01 day^{-1}), and T7 (0.07±0.01 day^{-1}), which were significantly different ($p<0.05$) from the SGR in T1 (0.04±0.01 day^{-1}), T3 (0.04±0.01 day^{-1}), and T2 (0.03±0.01 day^{-1}) as early as day 3 of culture period, however, decreases at the succeeding culture period.

3.2. Dry Weight and Cell Size

Figure 3 illustrates the dry weight (g L^{-1}) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results, the dry weights for T1, T2, T3, T4, T5, T6, T7, and T8 were 0.02±0.00 g L^{-1}, 0.00±0.00 g L^{-1}, 0.05±0.00 g L^{-1}, 0.06±0.01 g L^{-1}, 0.07±0.00 g L^{-1}, 0.09±0.00 g L^{-1}, 0.12±0.00 g L^{-1}, and 0.14±0.00 g L^{-1}, respectively. ANOVA revealed that T8 (0.14±0.00 g L^{-1}) was significantly increased ($p<0.05$) than other experimental treatments. Figure 4 reveals that the cell size (μm) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium varied significantly across treatments. Based on the results, the cell size for T1, T2, T3, T4, T5, T6, T7, and T8 were 8.20±0.06 μm, 8.57±0.21 μm, 10.28±1.00 μm, 10.60±0.81 μm, 22.90±3.50 μm, 26.14±1.86 μm, 25.95±0.92 μm, and 23.28±3.94 μm, respectively. ANOVA revealed that T5, T6, T7, and T8 were significantly increased ($p<0.05$) in size than T1, T2, T3, and T4. This indicates that the addition of AMPEP and BG-11, along with optimal NaCl concentrations, positively influences the cell size of *C. sorokiniana*, with T6 and T7 showing the most favorable conditions for growth.

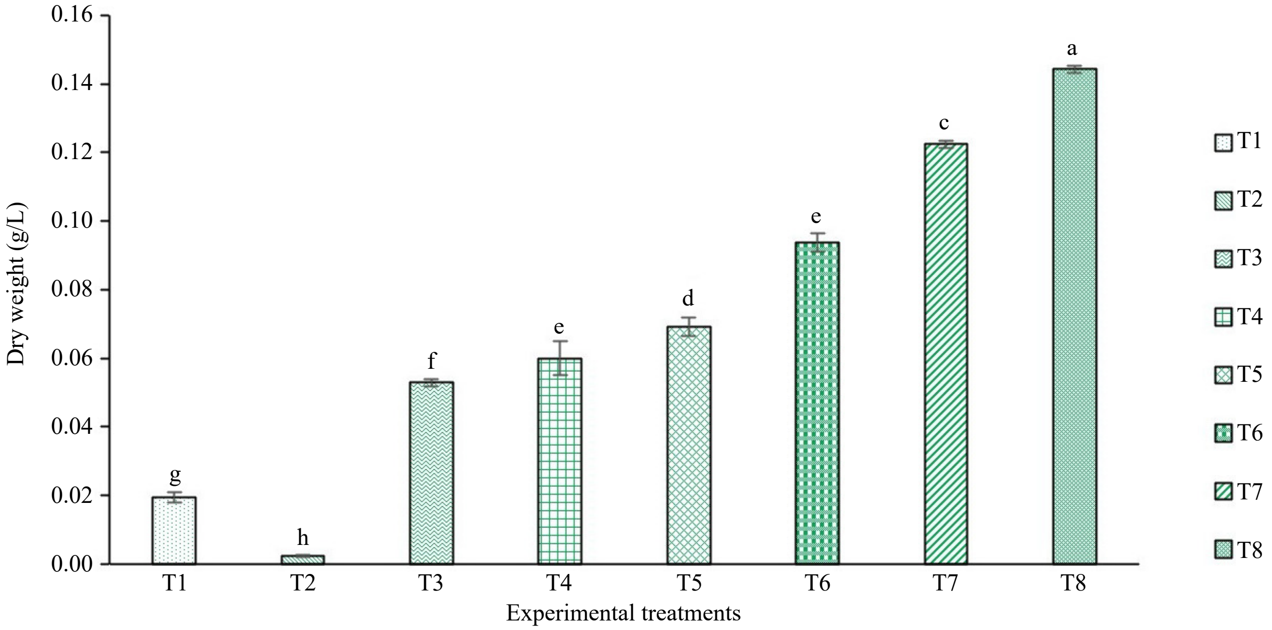

Figure 3. Dry weight (g L^{-1}) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

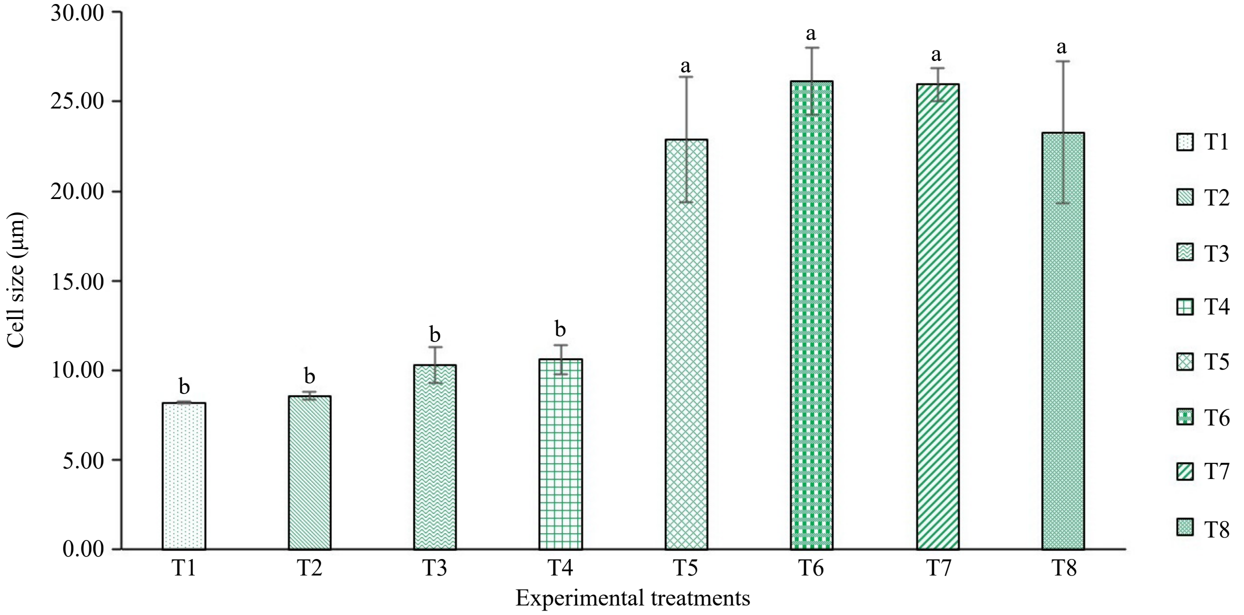

Figure 4. Cell size (µm) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

3.3. Chlorophyll a Pigment Accumulation

Figure 5 demonstrates the chlorophyll a pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results of the study, the chlorophyll a pigment accumulation in treatments T1, T2, T3, T4, T5, T6, T7, and T8 were 22.54±2.15 µg mL^{-1}, 0.87±0.28 µg mL^{-1}, 0.54±0.27 µg mL^{-1}, 1.13±0.42 µg mL^{-1}, 25.54±1.42 µg mL^{-1}, 21.06±0.72 µg mL^{-1}, 24.36±1.71 µg mL^{-1}, and 12.57±1.26 µg mL^{-1}, respectively. ANOVA revealed that T5 and T7 were significantly different ($p<0.05$) from T1. Additionally, Figure 6 illustrates the cellular chlorophyll a pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results of the study, the cellular chlorophyll a pigment accumulation in treatments T1, T2, T3, T4, T5, T6, T7, and T8 were 0.14±0.02 pg. $cell^{-1}$, 0.04±0.02 pg. $cell^{-}$

^{1}, 0.05±0.02 pg. cell^{-1}, 0.06±0.03 pg. cell^{-1}, 0.19±0.03 pg. cell^{-1}, 0.14±0.02 pg. cell^{-1}, 0.21±0.03 pg. cell^{-1}, and 0.15±0.01 pg. cell^{-1}, respectively. ANOVA revealed that T5 and T7 were significantly ($p<0.05$) different from T1, suggesting that nutrient supplementation positively influenced chlorophyll a accumulation at the cellular level.

3.4. Total Carotenoid Pigment Accumulation

Figure 7 illustrates the total carotenoid pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results, the total carotenoid content in T1, T2, T3, T4, T5, T6, T7, and T8 were 8.37±0.78 µg mL^{-1}, 0.45±0.08 µg mL^{-1}, 0.32±0.08 µg mL^{-1}, 0.61±0.11 µg

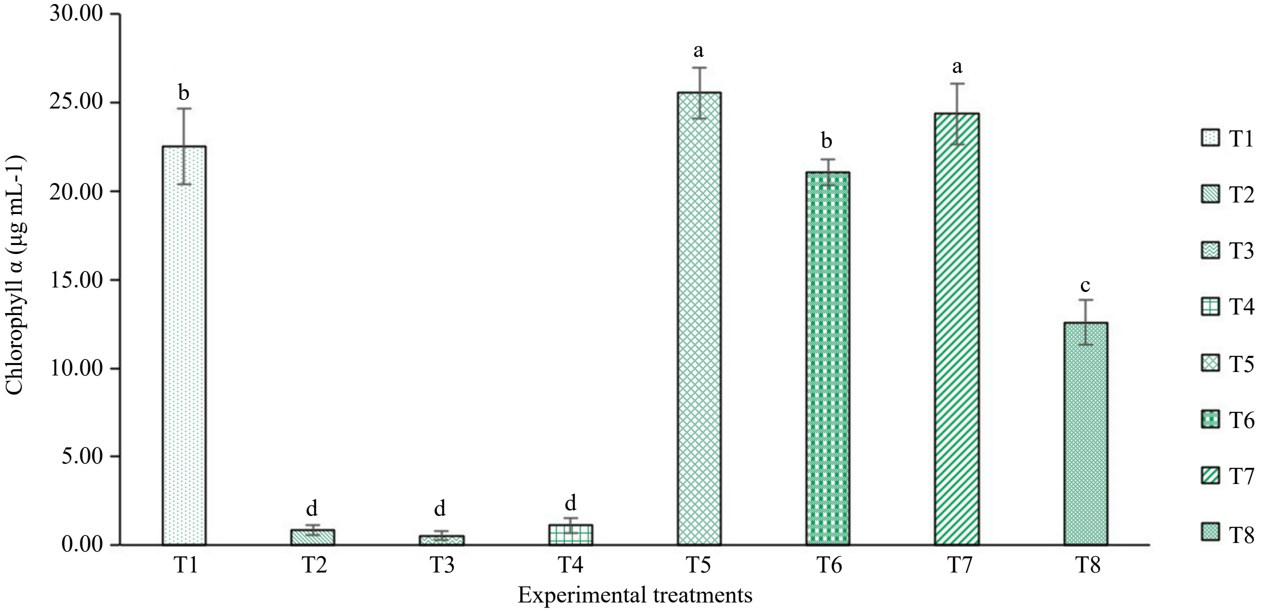

Figure 5. Chlorophyll a pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

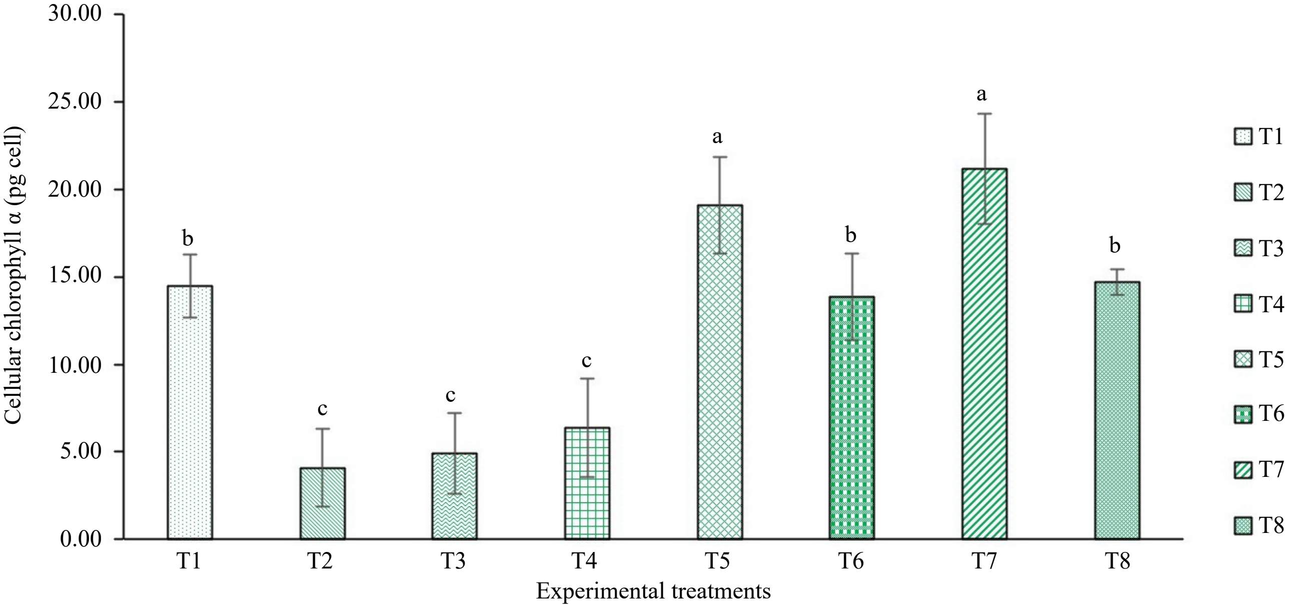

Figure 6. Cellular chlorophyll a pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

mL^{-1}, 9.97±0.88 µg mL^{-1}, 7.62±0.16 µg mL^{-1}, 8.67±0.63 µg mL^{-1}, and 4.35±0.40 µg mL^{-1}, respectively. ANOVA revealed that T5 is significantly different ($p<0.05$) from T6, T1, T7, T3, T2, T4. In addition, Figure 8 shows the cellular carotenoid accumulation (pg. cell^{-1}) of *C. sorokiniana* cultured under different experimental treatments. Based on the results, the cellular carotenoid content in T1, T2, T3, T4, T5, T6, T7, and T8 were 0.54±0.06 pg. cell^{-1}, 0.19±0.06 pg. cell^{-1}, 0.31±0.04 pg. cell^{-1}, 0.34±0.09 pg. cell^{-1}, 0.75±0.13 pg. cell^{-1}, 0.50±0.08 pg. cell^{-1}, 0.75±0.11 pg. cell^{-1} and 0.51±0.02 pg. cell^{-1}, respectively. ANOVA revealed that T5 and T7 exhibited significantly higher ($p<0.05$) carotenoid accumulation compared to T2.

3.5. Total Phenolic Content (TPC)

Figure 9 illustrates the TPC of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Based on the results, the TPC for T1, T2, T3, T4, T5, T6, T7, and T8 were 6.57±0.99 mg GAE g^{-1} DW, 5.91±0.21 mg GAE g^{-1} DW, 4.52±0.26 mg GAE g^{-1} DW, 5.28±0.10 mg GAE g^{-1} DW,

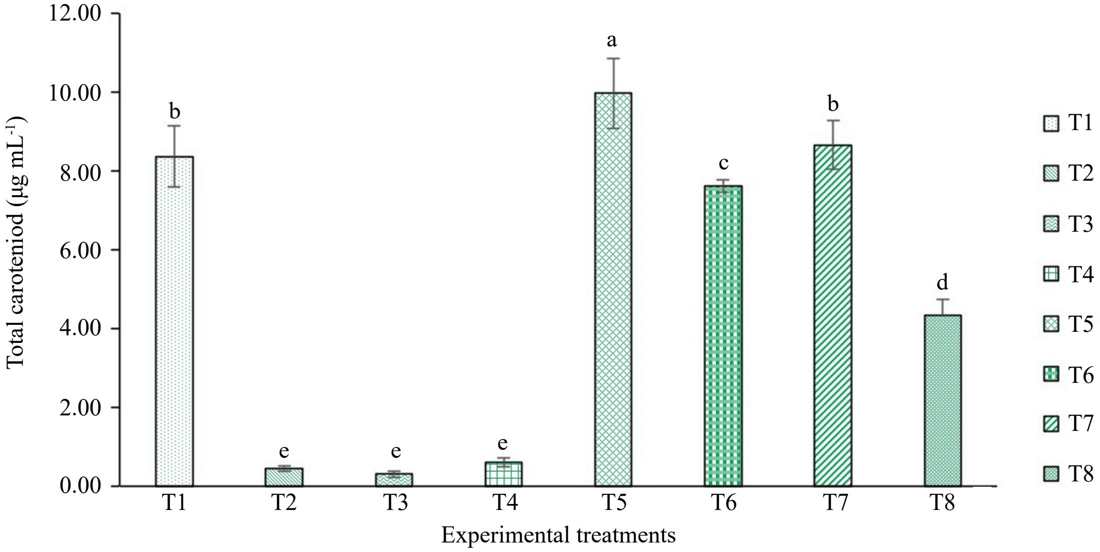

Figure 7. Total carotenoid (µg mL^{-1}) pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

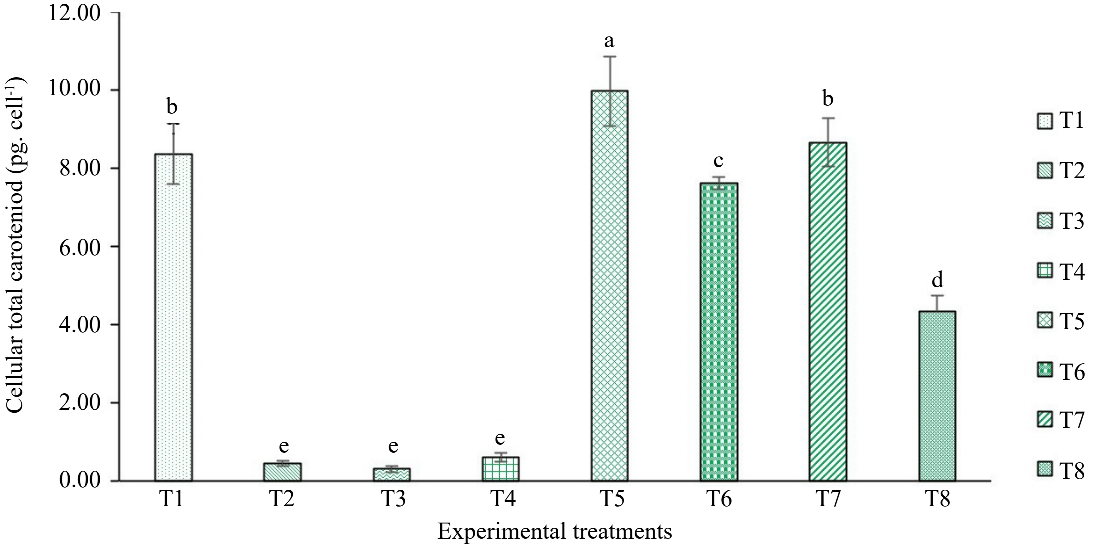

Figure 8. Cellular total carotenoid (pg. cell^{-1}) pigment accumulation of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

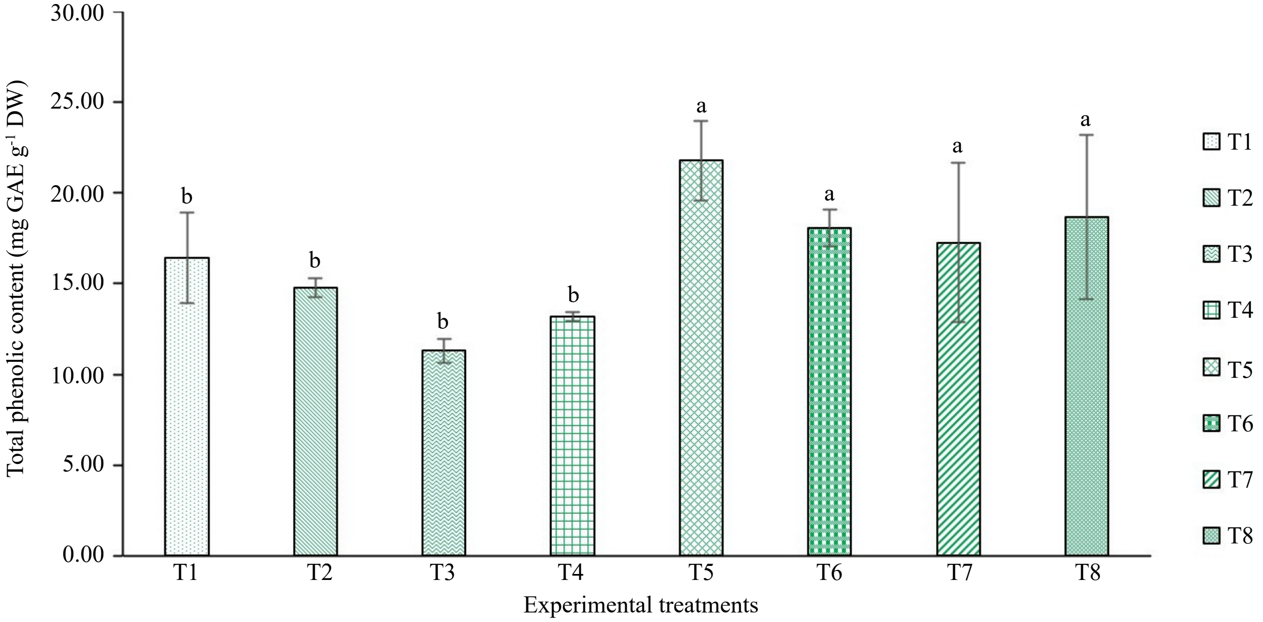

Figure 9. TPC (mg GAE g^{-1} DW) of *C. sorokiniana* cultured at different concentrations of NaCl, AMPEP, and BG-11 medium. Bar with the same letter are not significantly different ($p>0.05$). Values are in SEM (Standard Error Mean), n = 15

8.72±0.87 mg GAE g^{-1} DW, 7.23±0.40 mg GAE g^{-1} DW, 6.90±1.76 mg GAE g^{-1} DW, and 7.47±1.80 mg GAE g^{-1} DW, respectively. ANOVA revealed that T5, T6, T7, and T8 were significantly higher ($p<0.05$) compared to T1, T2, T3, and T4.

4. Discussion

4.1. Growth Response

Cell density, defined as the number of cells within a given space (Bryan *et al.* 2014; Sarri & Elp 2024), serves as a key indicator of algal growth. This study investigated the impact of varying NaCl concentrations, AMPEP (a plant growth regulator), and BG-11 medium on the cell density of *C. sorokiniana*. The results demonstrated significant variations in cell density across different treatment groups. The 15 g L^{-1} NaCl, 100 mg L^{-1} AMPEP, and 10 ml L^{-1} BG-11 medium, achieved the highest cell density at 15.92±2.04 × 10^6 cells mL^{-1} after 24 days of cultivation. This indicated that a combination of moderate salinity with optimal nutrient supplementation (AMPEP and BG-11) effectively enhanced microalgal growth. This finding aligns with the observations of Chokshi *et al.* (2017), who demonstrated that moderate salinity could act as a growth-promoting factor by enhancing osmotic regulation and metabolic efficiency in microalgae. Moreover, high cell densities were also observed in T5 and T7 suggesting a synergistic effect of AMPEP and BG-11 in supporting *C. sorokiniana* growth, as reported by Sarri *et al.* (2024a) and Sarri *et al.* (2024b). Moreover, treatments with low nutrient inputs or no supplementation, such as T1 and T3, exhibited significantly lower cell densities at 1.57 × 10^6 and 1.07 × 10^6 cells mL^{-1}, respectively. The relatively higher growth observed in T2 at 2.70 × 10^6 cells mL^{-1} compared to T1 emphasizes the importance of AMPEP even in the absence of BG-11. However, excessive salinity in T8 (25 g L^{-1} NaCl) led to reduced cell density at 8.50 × 10^6 cells mL^{-1}, likely due to osmotic stress and the inhibition of metabolic functions, consistent with the findings of Borowitzka (2018) and Sarri & Elp (2024), reported that high salinity levels can suppress microalgal growth by disrupting cellular homeostasis. These results underscore the critical importance of balancing nutrient supplementation and salinity levels to optimize the growth of *C sorokiniana.*

Specific growth rate (SGR) indicates how rapidly a population of microorganisms or cells is expanding (Berney *et al.* 2006; Sarri & Elp 2024). The present study investigated the SGR of *C. sorokiniana* under different concentrations of NaCl, AMPEP, and BG-11 medium. Among experimental treatments, T8 exhibited the highest SGR followed by T6. These two treatments showed significantly higher SGR values compared to T, T2, and T3, as early as day 3 of cultivation, suggesting an initial advantage in growth. However, a gradual decline in SGR was observed across all treatments over time. The reduction in SGR during later culture periods can be attributed to multiple factors, including nutrient depletion, the accumulation of inhibitory metabolites, and shifts in environmental conditions within the culture medium. Microalgal growth typically follows a lag, exponential, and stationary phase pattern, with the latter marked by a slowdown in growth due to nutrient exhaustion and the build-up of metabolic byproducts (Bolzan 2014). The variation in SGR across treatments underscores the intricate relationship between salinity, nutrient availability, and environmental conditions in regulating microalgal growth. While the elevated salinity levels in T6 and T8 initially promoted growth, excessive salt concentrations can induce osmotic stress and ion toxicity, ultimately hindering algal metabolism (Wei *et al.* 2017). The presence of AMPEP likely supplied essential organic nitrogen and phosphorus, key nutrients required for cellular biosynthesis and proliferation (Sarri *et al.* 2024a, 2024b). However, the optimal AMPEP concentration for *C. sorokiniana* may depend on the balance of other environmental factors, such as salinity levels and nutrient availability.

4.2. Dry Weight and Cell Size

Dry weight constitutes a critical parameter in evaluating microalgal biomass (Sarri *et al.* 2024a). It signifies the mass of the algal cells after complete water removal, offering a precise and reliable measure of the actual algal content (Zmora & Richmond 2003). The observed dry weight measurements of *C. sorokiniana* across varying concentrations of NaCl, AMPEP, and BG-11 medium reveal a clear trend as the concentrations of NaCl and AMPEP increase, there is a corresponding rise in biomass accumulation. Notably, T8, which contains the highest levels of NaCl (25 g L^{-1}) and AMPEP (200 mg L^{-1}), achieved the maximum dry weight of 0.14 g L^{-1}. These findings align with previous research indicating that *C. sorokiniana* can adapt to elevated salinity levels by accumulating biomass. Sarri *et al.* (2024b) investigated the effects of different AMPEP concentrations on *Chlorella* sp. cultures. They observed that a lower AMPEP concentration (125 mg L^{-1}) resulted in significantly higher cell densities and dry weight compared to control groups. Notably, the dry weight in the 125 mg L^{-1} AMPEP group was nearly double that of the control group. These findings suggest that AMPEP can be a potent growth promoter for *Chlorella* species, potentially by enhancing cell division and biomass accumulation. A study by Shen *et al.* (2020) demonstrated that supplementing cultures with glucose led to increased biomass and lipid yield in *C. sorokiniana*, suggesting that the alga can utilize alternative carbon sources to enhance growth under stress conditions. Similarly, research by Zhu *et al.* (2022) found that *C. sorokiniana* exhibited enhanced starch accumulation and growth rates under mixotrophic conditions, further supporting the alga's adaptability to varying environmental factors. The role of AMPEP in promoting biomass accumulation is also evident. Treatments incorporating AMPEP, like T5 through T8, displayed higher dry weights compared to those without it, implying that AMPEP may bolster the stress tolerance and metabolic activity of *C. sorokiniana*. This observation is consistent with findings by Ronga *et al.* (2019), who reported that certain biostimulants can enhance microalgal growth and biomass production under stress conditions. Furthermore, the combination of increased salinity and AMPEP appears to have a synergistic effect on biomass accumulation. This is in line with the work of Sambusiti *et al.* (2019), who observed that Nannochloropsis oculata exhibited higher dry weight at moderate salinity levels, indicating an adaptive response to saline environments.

Cell size in microalgae is a dynamic trait influenced by various environmental and physiological factors (Yap *et al.* 2016; Lim *et al.* 2022; Sarri *et al.* 2024b). The results of this study demonstrate a significant correlation between cell size in *C. sorokiniana* and the combined influence of NaCl, AMPEP, and BG-11 medium. Treatments characterized by lower cell sizes (T1-T4) strongly suggest that either a deficiency in essential nutrients or the presence of suboptimal growth conditions has impeded cellular expansion. Conversely, treatments incorporating higher concentrations of NaCl, AMPEP, and BG-11 (T5-T8) resulted in significantly larger cell sizes, indicating that these factors synergistically promote cellular development. This observation aligns with previous research findings (Singh *et al.* 2024), which have demonstrated that moderate salinity levels and nutrient supplementation can positively impact microalgal growth by enhancing cellular osmoregulation and metabolic activity. The most favorable conditions for maximizing cell size were observed in treatments T6 (26.14 μm) and T7 (25.95 μm), which incorporated 15-20 g L^{-1} NaCl, 100-150 mg L^{-1} AMPEP, and 10 mL L^{-1} BG-11. This suggests that a specific balance of salinity and nutrient supplementation creates an optimal environment for *C. sorokiniana* cell expansion. Elevated NaCl concentrations likely contribute to osmotically regulated cellular expansion (Halim *et al.* 2021; Sarri & Elp 2024), while AMPEP, a peptide-based growth enhancer, likely enhances photosynthetic efficiency and nutrient uptake (Hayashi *et al.* 2020; Sarri *et al.* 2024a, 2024b). BG-11 medium, a widely utilized algal growth medium, provides essential micronutrients and macronutrients, further supporting the cell's structural development (Durmaz & Erbil 2020; Erbil *et al.* 2021;

Elp *et al.* 2024; Sarri & Elp 2024; Erbil *et al.* 2024). However, a slight reduction in cell size was observed in T8 (23.28 μm), suggesting that excessive NaCl concentrations (25 g L^{-1}) may begin to exert significant osmotic stress, potentially counteracting the beneficial effects of AMPEP and BG-11.

4.3. Chlorophyll a Pigment

Chlorophyll a, the main pigment in microalgae, is essential for absorbing light and converting it into energy during photosynthesis. This directly influences how well the algae grow and produce biomass (Begum *et al.* 2016; da Silva & Lombardi 2020; Erbil *et al.* 2024). This study revealed a significant influence of NaCl concentration, AMPEP, and BG-11 medium on chlorophyll a accumulation in *C. sorokiniana*. The highest chlorophyll a content was observed in T5 (25.54 μg mL^{-1}), indicating that a balanced combination of salinity, growth enhancers, and essential nutrients optimizes pigment synthesis. Treatments lacking BG-11 medium (T2) or those with NaCl in the absence of sufficient nutrients (T3) exhibited significantly lower chlorophyll a level, highlighting the crucial role of these factors in pigment production. Furthermore, excessive NaCl (25 g L^{-1}) in T8 led to a substantial decrease in chlorophyll a content (12.57 μg mL^{-1}), suggesting that high salinity induces osmotic stress, negatively impacting photosynthesis and pigment synthesis. This aligns with previous research demonstrating that excessive salinity disrupts cellular ion balance, leading to oxidative stress and chlorophyll degradation in *Chlorella* species (Talebi *et al.* 2013; Sarri & Elp 2024). Treatments T6 (21.06 μg mL-1) and T7 (24.36 μg mL^{-1}) exhibited high chlorophyll a content, indicating that moderate NaCl (15–20 g L^{-1}) and AMPEP (100–150 mg L^{-1}) support photosynthetic activity. However, the decline in pigment accumulation in T8 suggests that the benefits of AMPEP may diminish under high-salinity conditions. While peptide-based growth stimulators like AMPEP enhance nutrient assimilation and photosynthetic efficiency, their effectiveness is contingent upon maintaining optimal salinity levels (Widman 2022). These findings corroborate previous research emphasizing the critical role of BG-11 medium in providing essential micronutrients for chlorophyll synthesis (Aslam *et al.* 2021; Pandey *et al.* 2023; Elbil *et al.* 2024) and the importance of carefully balancing salinity levels to prevent pigment degradation (Hu *et al.* 2018; Sarri & Elp 2024).

4.4. Total Carotenoid Pigment

Carotenoids function as auxiliary pigments in photosynthesis, broadening the spectrum of light wavelengths that can be utilized for energy conversion (Ke 2001; Hashimoto *et al.* 2016; Zia-Ul-Haq 2021). This study investigated the impact of varying NaCl concentrations, AMPEP (a plant growth regulator), and BG-11 medium on total carotenoid accumulation in *C. sorokiniana* culture. The results demonstrated significant variations in carotenoid content across different treatment groups. Treatment T5, with 10 g L^{-1} NaCl, 50 mg L^{-1} AMPEP, and 10 ml L^{-1} BG-11, exhibited the highest carotenoid content (9.97 μg mL^{-1}). This suggests that moderate NaCl levels, in conjunction with adequate nutrient supplementation, significantly enhanced carotenoid synthesis. This observation aligns with previous research by Ali *et al.* (2021) and Ren *et al.* (2021), which highlighted that optimal salt stress can stimulate the production of secondary metabolites, including carotenoids, as part of the microalgal oxidative stress response. Furthermore, Sun *et al.* (2023) reported that nutrient supplementation, particularly nitrogen, and phosphorus from BG-11 medium, plays a crucial role in regulating carotenoid biosynthesis by supporting photosynthetic efficiency and antioxidant mechanisms (Zhao *et al.* 2019; El-Fayoumy 2021; Song *et al.* 2022). Moreover, in the present study, the T2 and T3, lacking BG-11 medium, exhibited the lowest carotenoid levels (0.45 μg mL^{-1} and 0.32 μg mL^{-1}, respectively). This suggests that the absence of essential macronutrients and trace elements from BG-11 medium significantly impairs carotenoid synthesis. This finding is supported by Sarri & Elp (2024), who demonstrated that nutrient-deficient conditions in *Chlorella* sp. led to reduced carotenoid accumulation due to metabolic stress and disrupted biosynthetic pathways. Additionally, as observed in treatment T8 (25 g L^{-1} NaCl), excessive salinity significantly reduced carotenoid accumulation. This finding is consistent with studies by Gauthier *et al.* (2020) and Yang *et al.* (2024), which suggest that extreme salt concentrations disrupt cellular homeostasis, leading to oxidative damage and consequently decreased pigment synthesis.

4.5. Total Phenolic Content (TPC)

Phenolic compounds in microalgae, such as flavonoids, phenolic acids, tannins, lignans, and coumarins, are secondary metabolites that play diverse roles, particularly in responding to environmental stresses such as heavy metals, salinity, and temperature variations (Del Mondo *et al.* 2022). This study investigated the influence of varying NaCl concentrations, AMPEP (a plant growth regulator), and BG-11 medium on the TPC of *C. sorokiniana* culture. The results demonstrated significant variations in TPC across different treatment groups. The T5, with 10 g L^{-1} NaCl, 50 mg L^{-1} AMPEP, and 10 mL L^{-1} BG-11, exhibited the highest TPC (8.72 mg GAE g^{-1} DW) than other experimental treatments, suggesting that the presence of BG-11 medium and AMPEP supplementation played a crucial role in enhancing phenolic compound accumulation. This finding aligns with previous studies, such as Zhao *et al.* (2019) and Paterson *et al.* (2023), which reported that nutrient-rich conditions, particularly the availability of nitrogen and trace elements, positively influence phenolic compound biosynthesis in microalgae. Similarly, Goiris *et al.* (2015) and Cichoński & Chrzanowski (2022) investigated that optimal nutrient supplementation enhances secondary metabolite production, including phenolics, which contribute to antioxidant activity. The increased TPC in T5 can be attributed to the synergistic effect of moderate salinity and nutrient availability, as supported by Uzlasir *et al.* (2023), who demonstrated that controlled salt stress combined with sufficient macronutrients enhances phenolic compound accumulation as a protective response against oxidative stress. Furthermore, in the present study, the T3, lacking both AMPEP and BG-11, exhibited the lowest TPC (4.52 mg g^{-1}), emphasizing the critical role of nutrient supplementation in phenolic biosynthesis. This observation is consistent with the findings of Suparmaniam *et al.* (2023) and Eilam *et al.* (2023), who reported that microalgae cultivated under nutrient-deficient conditions exhibit lower phenolic content due to impaired secondary metabolite pathways. Additionally, excessive salinity, as observed in T8 (25 g L^{-1} NaCl), did not result in the highest TPC. This suggests that extreme salt stress may have detrimental effects on phenolic synthesis, as also observed by Kusvuran & Can (2020) and Sarsekeyeva *et al.* (2024), who found that excessive osmotic stress can negatively impact microalgal metabolism (Shetty *et al.* 2019).

In conclusion, this study investigated the combined effects of salinity and AMPEP concentrations on the growth, pigment accumulation, and total phenolic content of *Chlorella sorokiniana* culture. The results demonstrated that 15 g L^{-1} salinity with 100 mg L^{-1} AMPEP significantly enhanced cell density, while 25 g L^{-1} salinity with 200 mg L^{-1} AMPEP improved the specific growth rate and dry weight. Additionally, 15 g L^{-1} salinity with 100 mg L^{-1} AMPEP and 20 g L^{-1} salinity with 150 mg L^{-1} AMPEP led to an increase in cell size. For pigment accumulation, the highest chlorophyll a and total carotenoid levels were recorded at 10 g L^{-1} salinity with 50 mg L^{-1} AMPEP. This same combination also resulted in the highest total phenolic content. These findings indicate that optimizing salinity and AMPEP concentrations in the nutrient medium can effectively enhance growth performance, pigment production, and biochemical composition in *Chlorella sorokiniana* culture. This study provides valuable insights for improving microalgae cultivation, particularly for applications in aquaculture, biotechnology, and bioresource industries.

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