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Impact of Acadian Marine Plant Extract Powder (AMPEP) concentration in nutrient medium on the growth and lipid accumulation of *Chlorella* sp. Culture

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ABSTRACT
Microalga of the genus *Chlorella* has developed a stable industry as dietary supplements for humans and animals based on their scientific and commercial interests. The growth of *Chlorella* sp. cultures has been enhanced by using a variety of nutrients to improve pigmentation, lipid content, and growth. Acadian Marine Plant Extract Powder (AMPEP) improves crops and macroalgae production, such as seaweeds. However, AMPEP has not yet been studied as a means of producing microalgae. Therefore, this study investigates microalgae production in a nutrient medium containing AMPEP. Three concentrations of AMPEP were prepared: group A (125 mg L^{-1} AMPEP), group B (625 mg L^{-1} AMPEP), and group C (0 mg L^{-1} AMPEP) as control. Experiments were conducted for each group for 21 days in triplicate. Results revealed that lower AMPEP (125 mg L^{-1}) concentration added to the nutrient medium provides higher cell densities in *Chlorella* sp. culture. 125 mg L^{-1} AMPEP in a nutrient medium reached the highest cell density of 1.28 fold cell mL^{-1} than the control group. Additionally, the dry weight of groups A, B, and C were calculated as 2.57 ± 0.12 g L^{-1}, 1.37 ± 0.06 g L^{-1}, and 1.58 ± 0.16 g L^{-1}, respectively. The cell sizes of groups A, B, and C were 4.80 ± 1.32 µm, 5.20 ± 1.87 µm, and 3.80 ± 0.79 µm, respectively. Moreover, the highest level of lipid accumulation of *Chlorella* sp. culture was achieved by group B with a lipid content of 10.44 ± 1.28 %, followed by group A with a lipid content of 8.55 ± 0.80 %, which was higher than the control group (group C) with a lipid content of 7.04 ± 0.93 %. Hence, the present study shows that AMPEP used in microalgae production may improve growth and lipid accumulation.

Keywords: AMPEP, *Chlorella* sp., Growth, Pigments, Lipids

1. Introduction

Microalgae are nutrient-rich organisms because of their biochemical composition, particularly their lipid and fatty acid composition (Niccolai et al. 2019). The changes in culture conditions, including medium type, temperature, and light, can significantly affect biochemical composition (Durmaz et al. 2008; George et al. 2014). Microalgae as direct feed in larval tanks is essential in aquaculture for bivalves at all growth stages and for some crustacean and fish species larvae (Kaparapu 2018). In aquaculture, zooplankton transports nutrients from microalgae to larger culture aquatic organisms (Vismara et al. 2003; Montemezzani et al. 2015). In addition, the microalgae industry produces various biotechnological products, such as feed, natural colorants, health foods, bioenergy, and pharmaceuticals (Rizwan et al. 2018; Rahman 2020).

Chlorella sp. is one of the most widely produced microalgae due to its high productivity, cellular composition, and ability to grow photoautotrophically, heterotrophically, and mixotrophically (Caporgno et al. 2019; Erbil & Durmaz 2020). Researchers stated that *Chlorella* sp. contains high levels of pigments, fatty acids, lipids, protein, minerals, and vitamins (Silva et al. 2020; Tayemeh et al. 2020). Microalgae from the genus *Chlorella* are distinguished by the two main types of chlorophylls (*a* and *b*), which are their most abundant pigments, as well as other pigments such as astaxanthin, c-astaxanthin, beta-carotene, and lutein that contribute to their pigment composition (Christaki et al. 2015; Khanra et al. 2018; Silva et al. 2020). The amount of pigments involved in light harvesting depends on the growth conditions, especially the light intensity, the salinity, and the nutrients available (Juneja et al. 2013). Additionally, it has been found that microalgae, mainly green algae, have high levels of lipids, although their productivity varies considerably depending on the species of algae (Griffiths et al. 2012; Wahidin et al. 2013; Khan et al. 2018). It has been found that *Dunaliella*, *Scenedesmus*, and *Chlorella* species have the highest levels of lipids in their cells, ranging from a lipid content of 10 to 67% (Islam et al. 2013; Nascimento et al. 2013; Udayan et al. 2023). It is well

documented that nutrient limitation creates stress conditions in cells, which in turn enhances lipid accumulation in microalgae (Sulochana & Arumugam 2020).

Ascophyllum nodosum is a brown seaweed containing bioactive substances that may influence the molecular, biochemical, and physiological function of crop plants (Di Stasio et al. 2018; Shukla et al. 2019). Several temperate regions worldwide, such as the United Kingdom, Iceland, Ireland, Norway, France, and Canada, where *Ascophyllum nodosum* seaweed thrives abundantly (Hurtado et al. 2009). Additionally, an extract from the brown macroalga *A. nodosum* known as *Ascophyllum* (Acadian) Marine Plant Extract Powder (AMPEP) has been studied for micropropagation and field cultivation of seaweeds, particularly *Kapphycus* species (Hurtado & Critchley 2018; Silva et al. 2019). This extract is widely applied to increase the performance of land crops and has been reported to enhance the growth of some seaweed crops (Umanzor et al. 2019). The use of AMPEP has improved macroalgae growth, decreased diseases, and increased carrageenan quality (Loureiro et al. 2017; Tahiluddin et al. 2022). However, no studies have been conducted on using AMPEP to produce microalgae. Thus, this study aims to investigate the effect of the AMPEP concentration in nutrient medium on microalgae production.

2. Material and Methods

2.1. Microalgae

Freshwater microalga *Chlorella* sp. was used in this study and obtained from the Aquaculture Department, Faculty of Fisheries, Kastamonu University, Türkiye.

2.2. Culture condition

500 mL flat-bottom flasks were used in *Chlorella* sp. culture. BG-11 medium was used as a nutrient medium (Tables 1 and 2). Afterward, AMPEP was added to the flask at different concentrations. The composition of AMPEP is shown in Table 3. Each solution was autoclaved for 20 minutes at 121°C. After autoclaving, experimental groups were inoculated at an initial density of 1.0 x 10^6 cells mL^{-1}. Cultures were made with artificial lighting in the laboratory environment. Fluorescent lamps (MASTER TL-D Super 80 36W/865 1SL/25) were used for lighting. The ventilation of the cultures was carried out with an air motor, and syringe filters with an opening of 0.2 µ were used to prevent contamination. The air conditioner maintained a temperature of 20±1 °C.

Table 1- BG-11 nutrient medium (Erbil et al. 2021)

Solution A	***For 500 mL***
$NaNO_3$	*75.0 g*
Solution B	***For 500 mL***
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	3.75 g
$CaCl_2.2H_2O$	1.80 g
Citric acid	0.30 g
Ammonium ferric citrate green	0.30 g
$EDTANa_2$	0.05 g
Na_2CO3	1.00 g

Table 2- Trace element composition

Trace element solution	***For 1 000 mL***
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.22 g
$Na_2MoO_4.2H_2O$	0.39 g
$CuSO_4.5H_2O$	0.08 g
$Co(NO_3)_2.6H_2O$	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)

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%

2.3. Experimental design

AMPEP was obtained from the Mindanao State University-Tawi-Tawi College of Technology and Oceanography (MSU-TCTO) Sanga-Sanga, Bongao, Taw-Tawi Philippines. The AMPEP was added to the flask with a nutrient medium, as given in Table 4. The control group does not contain any AMPEP source. The experiment was done in triplicates.

Table 4- Experimental group and AMPEP concentration

Experiment	***Unit***	***AMPEP Concentration***
Group A	mg L^{-1}	125
Group B	mg L^{-1}	625
Group C (Control)	mg L^{-1}	0

2.4. Growth analysis

Every three days, samples of microalgae were collected for cell counting and analysis. A Neubauer hemocytometer was used to count cells daily under the light microscope, and contamination was checked on a regular basis visually. 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 group in an oven at 105 °C for 2 hours (Erbil et al. 2021). The specific growth rate (μ) was calculated by the following below.

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

Where: N_2 is the biomass cell number at the time (t_2). N_1 is the beginning biomass cell number at a time (t_1).

2.5. Cell sizes measurement

Photographs of cells were taken and then transferred to ImageJ Software (National Institutes of Health, USA), where measurements were conducted for each group of cells.

Lipid Analysis

A spectrophotometric method was followed by Mishra et al. (2014) to determine the total amount of lipids. A phospho-vanillin and sulfuric acid solution were used as a reagent in determining the lipid amount of microalgae.

Statistical Analysis

IBM SPSS software version 20 was used to analyze the collected data of growth and lipid accumulation of *Chlorella* sp. culture at $P<0.05$ significance level. Data were presented as mean ± standard error of the mean (SEM). Determination of significant differences was computed through the One-way Analysis of Variance (ANOVA). Levene's Test was used to test for homogeneity of variance, and Duncan's Post-Hoc Test was used to rank the mean (Hairol et al. 2022; Sanuddin et al. 2023).

3. Results

3.1. Growth

The experiment was started at an initial density of 1.0 x 10^6 cell mL^{-1} and was continued for 21 days of culture. 125 mg L^{-1} (group A) and 625 mg L^{-1} (group B) concentrations of AMPEP were used in the medium, and no AMPEP concentration was referred to as control (group C). Figure 1 shows the cell number of *Chlorella* sp. culture at different concentrations of AMPEP in a nutrient medium. Results revealed that the maximum cell number of groups A and B reached 146.00 ± 20.51 x 10^6 cell mL^{-1} and 73.42 ± 9.39 x 10^6 cell mL^{-1}, respectively, after 21 days of culture. The maximum cell number of the control group (group C) reached 113.79 ± 9.41 x 10^6 cell mL^{-1} at 21 days of culture. Group A (125 mg L^{-1}) cell growth was statistically higher ($P<0.05$) from group B (625 mg L^{-1}) and control group (group C). The maximum specific growth rates (SGR) were obtained at six days of culture for all experimental groups. SGR of groups A, B, and C were 0.60 ± 0.06 day^{-1}, 0.29 ± 0.12 day^{-1}, and 0.61 ± 0.08 day^{-1}, respectively, of which 125 mg L^{-1} concentration of AMPEP in a nutrient medium and control group were significantly higher than 625 mg L^{-1} concentration of AMPEP (Figure 2). Moreover, it was observed that the cell sizes of groups A, B, and C were 4.80 ± 1.32 µm, 5.20 ± 1.87 µm, and 3.80 ± 0.79 µm, respectively, of which no significant differences ($P<0.05$) were observed among experimental groups (Figure 3). The maximum dry weight of groups A, B, and C were calculated as 2.57 ± 0.12 g L^{-1}, 1.37 ± 0.06 g L^{-1}, and 1.58 ± 0.16 g L^{-1}, respectively. There was a significant difference ($p>0.05$) observed among the experimental group, of which a lower concentration of AMPEM (125 mg L^{-1}) produced higher dry weight than the higher concentration of AMPEP (125 mg L^{-1}) and the control group (Figure 4). In terms of cellular dry weight, groups A, B, and C achieved 20.04 ± 4.27 pg. $cell^{-1}$, 18.86 ± 2.36 pg. $cell^{-1}$, and 14.06 ± 1.03 pg. $cell^{-1}$, respectively, of the cellular dry weight of *Chlorella* sp. culture (Figure 5).

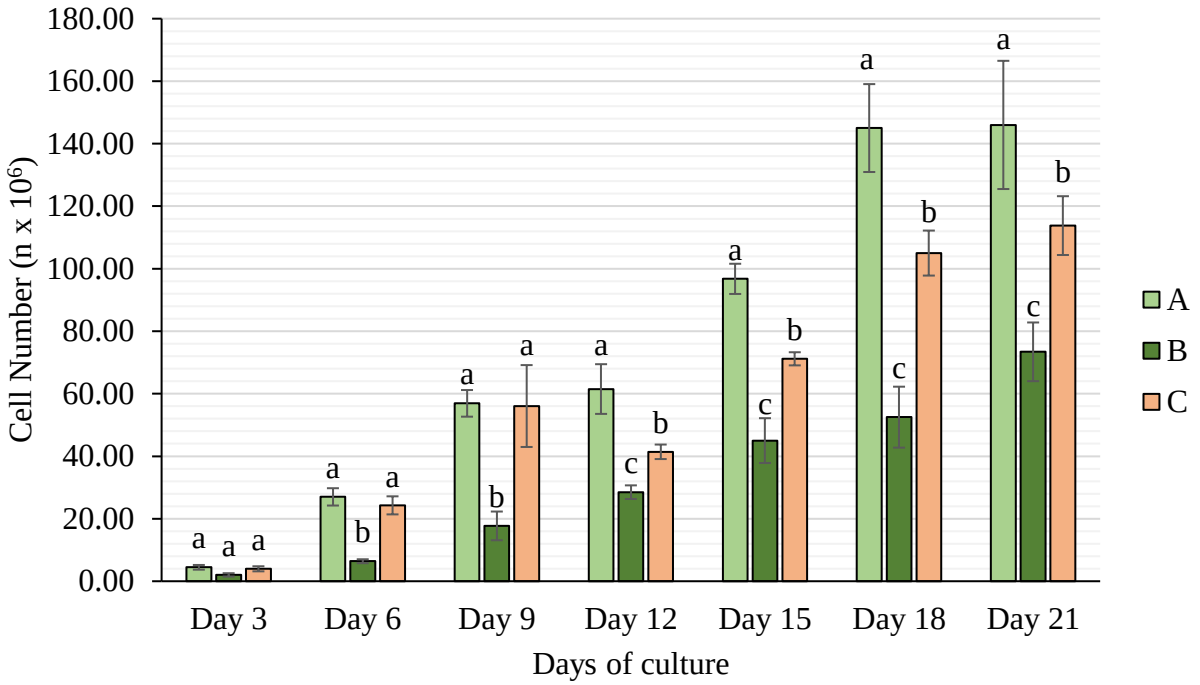

Figure 1- Cell number (n x 10^6) of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different ($P<0.05$). Mean error bars are in STD (standard deviation).

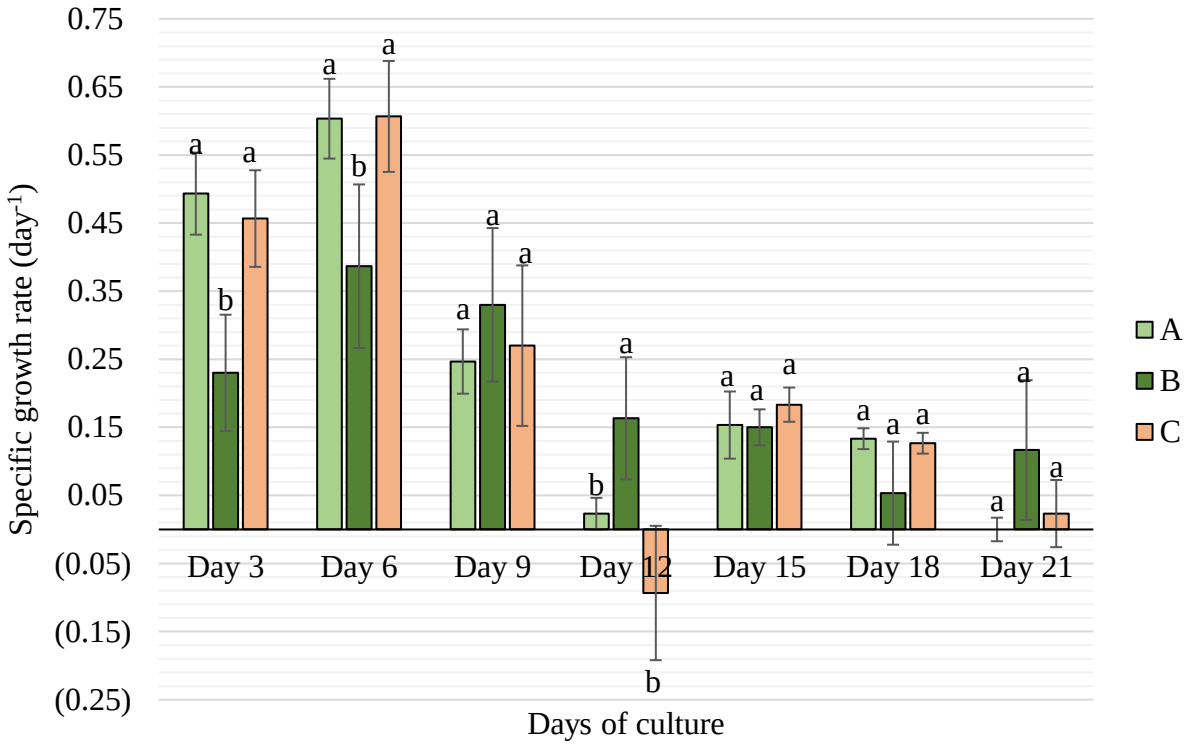

Figure 2-The specific growth rate of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different (P<0.05). Mean error bars are in STD (standard deviation).

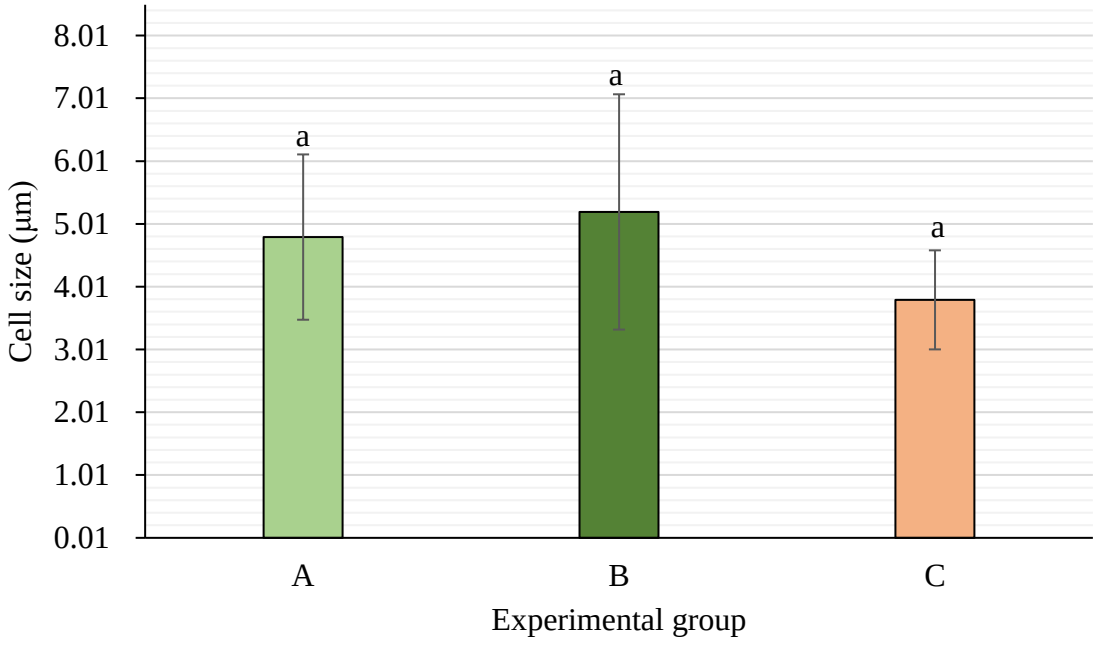

Figure 3- Cell size (µm) of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different (P<0.05). Mean error bars are in STD (standard deviation), n=30.

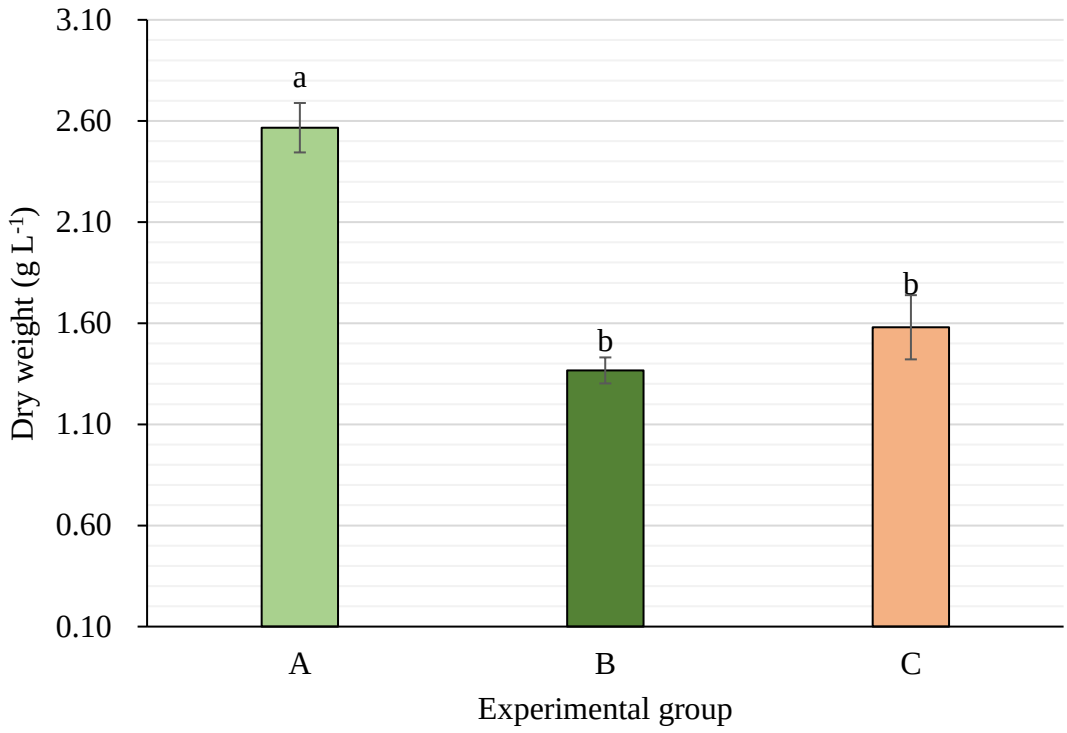

Figure 4- Dry weight (g L^{-1}) of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different (P<0.05). Mean error bars are in STD (standard deviation).

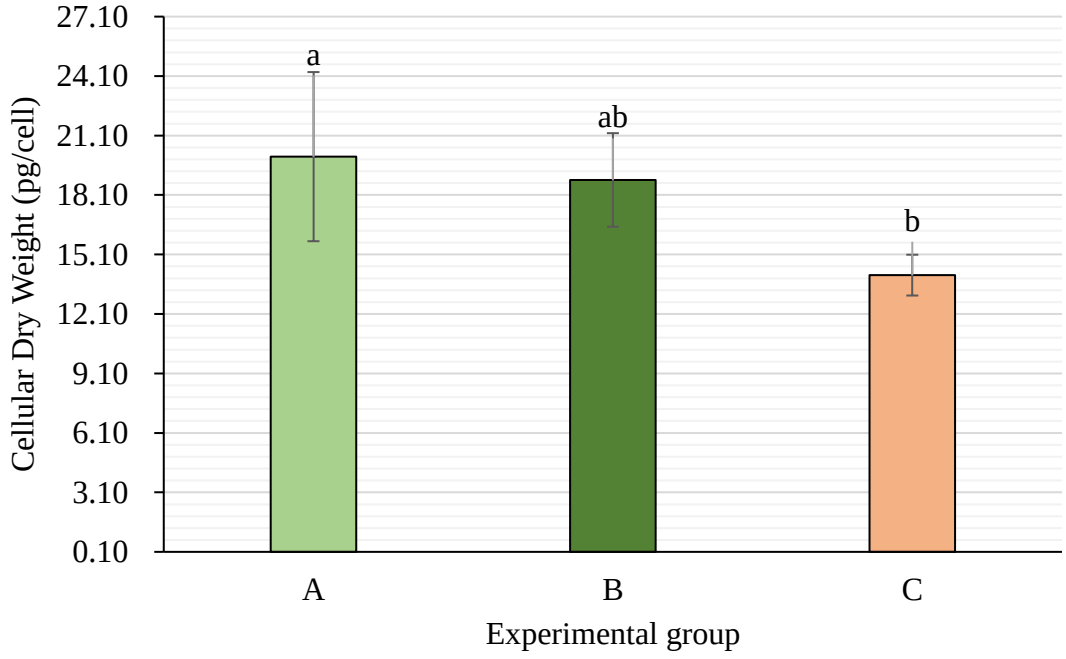

Figure 5- Cellular dry weight (pg. $cell^{-1}$) of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different (P<0.05). Mean error bars are in STD (standard deviation).

3.2. Lipid

Figure 4 shows lipid accumulation of *Chlorella* sp. culture at different concentrations of AMPEP in a nutrient medium. The measurement of lipid accumulation was done in triplicates. The maximum lipid content of 625 mg L^{-1} (group B) concentration of AMPEP in the nutrient medium was 10.44 ± 1.28%, while the maximum lipid content of 125 mg L^{-1} (group B) concentration of AMPEP (group A) was 8.56 ± 0.80%. The maximum lipid content in the control group (group C) reached 7.04 ± 0.93%. The lipid content showed no significant difference ($P>0.05$) observed between 125 mg L^{-1} (group A) and 625 mg L^{-1} (group B) of AMPEP added to *Chlorella* sp. culture. However, the lipid content of the control group (group C) was recorded as the lowest. A significant difference ($P<0.05$) was observed between the control group and 625 mg L^{-1} (group B) of AMPEP concentration added to *Chlorella* sp. culture.

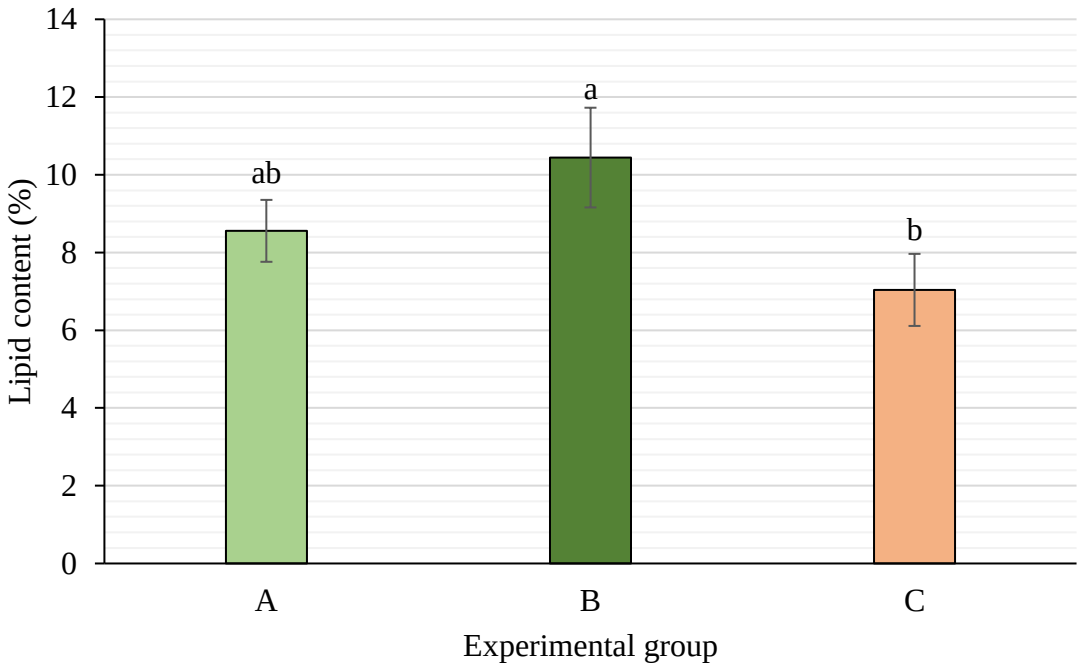

Figure 4- Lipid content (%) of *Chlorella* sp. culture at different concentrations of AMPEP. Group A (125 mg L^{-1}), group B (625 mg L^{-1}), and group C (Control). Differences in the letters are significantly different ($P<0.05$). Mean error bars are in STD (standard deviation), n=9.

4. Discussion

Many crops and horticulture plants have been successfully treated with seaweed extracts to alleviate biotic and abiotic stress (Hurtado & Critchley 2018). An extract from the brown macroalga *A. nodosum* known as *Ascophyllum* (Acadian) Marine Plant Extract Powder (AMPEP) is widely used to increase the performance of crops and horticulture plants, as well as to enhance macroalgae growth such as seaweeds (Silva et al. 2019; Umanzor et al. 2019; Tahiluddin et al. 2022). The present study investigates the use of AMPEP in microalga *Chlorella* sp. culture. According to the results of this study, AMPEP addition to the culture medium of *Chlorella* sp. culture provides higher cell densities. The lower concentration of AMPEP (125 mg L^{-1}) reached higher cell density than groups B and C. It is thought that the brownish color of AMPEP might be reducing the light transmittance, which could be the reason for the difference in growth parameters between AMPEP groups.

To date, various organic and inorganic promoters have been used in microalgae culture, and it has been determined that they induce improvements in culture parameters. For instance, the use of myo-inositol with 500 mg L^{-1} concentration in the *Nannochloropsis oculata* culture resulted in 1.28 fold cells per mL than the control group (Erbil et al. 2020). Another study found that adding 500 mg L^{-1} of myo-inositol to the microalga *Dunaliella salina* culture led to an increase of 1.4-fold cell mL^{-1} over the control group (Cho et al. 2015). Many studies have been conducted on microalgae that grow well in different culture media. Using different phosphate concentrations in a nutrient medium on a *Chlorella vulgaris* culture, a cell number of 2.38 x 106 cells mL^{-1} was obtained (Chia et al. 2013). Additionally, the effect of the phosphate source in an F/2 medium reached a cell number of 32 × 106 cell mL^{-1} on *Chlorella* sp. culture (Aziz & Siti Mariam 2016). Moreover, a study conducted by Durmaz et al. (2007) determined that microalga *Nannochloropsis oculata* could use NO^{-3} as its sole nitrogen source and obtained a maximum cell density of 52 ± 0.3 x 106 cell mL^{-1}. Our results revealed that the lower concentration of AMPEP in a culture medium positively increased the cell density of the *Chlorella* sp. culture.

A general approach to determining growth-limiting substances in cell cultures involves determining their specific growth rate (SGR). One of the factors that can influence microalgae growth is nutrient availability (Jaiswal et al. 2020). In the present study, a lower concentration (125 mg L^{-1}) of AMPEP in a nutrient medium obtained a maximum SGR of 0.60 day^{-1} of *Chlorella* sp. culture. As reported by Liu et al. (2021), phosphate and iron sources in a nutrient medium produced an SGR of 0.286 day^{-1} in the growth of *Chlorella vulgaris* culture, which is lower than the present study such as in the experimental group A (125 mg L^{-1}). Additionally, the SGR of all experimental groups in the present study was higher than the study of Erbil et al. (2021), which examined the production of *Chlorella* sp. culture utilizing BG-11 medium and obtained an SGR of 0.078 day^{-1}. Hence, using BG-11 medium with the addition of AMPEP increased the SGR of *Chlorella* sp. culture.

The yield of *Chlorella* cells cultured with AMPEP was estimated by measuring *Chlorella* biomass after 21 days of cultivation. In the present study, there significant differences were observed among the experimental group, of which the lower concentration of AMPEM (125 mg L^{-1}) produced a higher dry weight of 2.57 g L^{-1} than the dry weight of a higher concentration of AMPEP (625 mg L^{-1}) and the control group. There was a significant amount of dry weight generated in the present study compared to the amount of algae in open raceway ponds, which usually ranges between 0.1 and 0.5 g L^{-1} but can reach up to 1.4 g L^{-1} (Ketheesan & Nirmalakhandan 2012; Ashokkumar et al. 2014; Kumar et al. 2015; Zhu 2015). The present study was comparable to other researchers, which cultured microalga *Nannochloropsis oculata* on fiberglass reinforced plastic panel photobioreactor enriched with f/2 medium and achieved a dry weight of 0.81 g L^{-1} (Durmaz & Erbil 2020). In another study, it was reported that the productivity of continuous cultures enriched with f/2 medium reached 2.02 and 3.03 g L^{-1} at helical tubular photobioreactors (Briassoulis et al. 2010). The results of their study were comparable to those of the present study, in which lower AMPEP levels in a nutrient medium led to an increase in dry weight in *Chlorella* sp. cultures. Moreover, the present study exceeded the results of Feng et al. (2012), in which *Chlorella zofingiensis* was cultivated on BG-11 medium (enriched with nitrogen and phosphate) and achieved a dry weight of 0.90 g L^{-1}. Thus, using BG-11 medium with the addition of AMPEP increased the dry weight of *Chlorella* sp. culture.

Several factors affect the growth and accumulation of lipids in microalgae, including nutrient concentrations, carbon dioxide aeration, and other sustainability-related factors (Xin et al. 2010). As a result of the present study, the incorporation of AMPEP in the nutrient medium increased the lipid production of *Chlorella* sp. culture. In other study, *Chlorella vulgaris* was cultivated in f/2 medium achieved a lipid content of 7.80% (Liu et al. 2008). Comparatively, it is lower than the present study in which higher and lower concentrations of AMPEP achieved lipid content of 10.44% and 8.56%, respectively. Moreover, studies have shown that nutrient limitations can result in high lipid content but low biomass levels (Liu et al. 2021; Hasnain et al. 2023). Low phosphate concentrations in a BG-11 nutrient medium have shown that it lead to greater lipid accumulation of *Chlorella pyrenoidosa* culture (Rana et al. 2020). In the present study, a high level of AMPEP in a BG-11 medium increased lipid accumulation in *Chlorella* sp. culture. Hence, the presence of AMPEP concentration in a nutrient medium may lead to high levels of lipid accumulation of *Chlorella* sp. culture.

5. Conclusions

Chlorella sp. is very important in aquaculture hatcheries due to its properties (pigments, lipids, cell size, fatty acids, etc.). The present study investigated the use of different concentrations of AMPEP in the production of microalga *Chlorella* sp. culture. As a result of the study, a lower concentration of AMPEP (125 mg L^{-1}) in a nutrient medium significantly improves the growth of *Chlorella* sp. culture. Additionally, both concentrations of AMPEP (125 mg L^{-1} and 625 mg L^{-1}) in a nutrient medium increased the lipid accumulation of *Chlorella* sp. culture. However, this study requires further analysis, such as other biochemical compositions (pigments, fatty acids, proteins, etc.), as well as optimization of AMPEP concentrations in the production of microalga *Chlorella* sp. culture.

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