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Research Article

Effect of whey powder on the biomass, biochemical, and pigment composition of *Haematococcus pluvialis* and *Scenedesmus* sp.

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Abstract

This study investigated the effects of whey powder (WP), used as a nutrient medium, on the biomass production, biochemical composition, and pigment content of *Haematococcus pluvialis* and *Scenedesmus* sp. The experimental design was set up with four groups and all groups had three replicates. The control group (C) contained only Bold Basal Medium with 3-fold nitrogen and vitamins (3N-BBM+V) without WP. The second experimental group was formulated as 3N-BBM+V with 5 g L^{-1} WP (W5), the third group as 3N-BBM+V with 10 g L^{-1} WP (W10), and the fourth group as 3N-BBM+V with 15 g L^{-1} WP (W15). In this study, the highest mean biomass was obtained as 0.88 g L^{-1} for *H. pluvialis* (WP10) and 0.45 g L^{-1} for *Scenedesmus* sp. (WP15). The maximum total lipid productivities of *H. pluvialis* and *Scenedesmus* sp. were recorded as 43% (w/w, dry weight) in the WP5 group and 66% (w/w, dry weight) in the WP10 group, respectively. Under mixotrophic conditions, the highest protein contents were obtained in WP10 for *H. pluvialis* (20.94 mg mL^{-1}) and in WP15 for *Scenedesmus* sp. (44.94 mg mL^{-1}). Astaxanthin, carotenoid and phycocyanin productivity of *H. pluvialis* and *Scenedesmus* sp. was highly determined under mixotrophic conditions. In conclusion, the present study demonstrates that the supplementation of the culture medium with whey powder significantly enhances both biomass production and biochemical composition. Therefore, whey powder can be recommended as an effective supplementary nutrient source for the cultivation of these microalgae.

Introduction

Microalgae are a natural food source that can produce many important metabolites such as proteins, carbohydrates, lipids, vitamins, minerals, and pigments. Recently, the use of microalgae in food, cosmetics, and bioenergy has become an increasingly important issue (Ang *et al.*, 2024). In addition, microalgae can store omega-3 and omega-6 fatty acids, which are important unsaturated fatty acids in their cells. They are also used in the food industry as functional food and natural colorants and astaxanthin (Karabulut and Gulay, 2016). These microorganisms, which can develop under autotrophic, heterotrophic, and mixotrophic conditions, contain 4-55% lipid, 6-57% carbohydrate, and 10-63% protein (Singh *et al.,* 2011; Elcik and Cakmakcı, 2017). Although microalgae are valuable in terms of their nutritional composition, they are difficult to cultivate commercially due to their high production costs.

Different green microalgal species produce 30–50% of the total lipid content of dry cells under stress conditions (Tripathi *et al.*, 2015). Fatty acids in microalgae are composed of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-linoleic acid (GLA), triglycerides (TAG), polyunsaturated fatty acids (PUFA), and arachidonic acid (ARA). PUFAs are found at higher concentrations in green algae than in other groups (Bigogbo *et al.*, 2002). However, the total lipid productivity of microalgae depends on both the biotic and abiotic conditions.

Microalgae exhibit several photosynthetic acclimation strategies under changes of abiotic (light, pH, salinity, etc.) conditions (Wacker *et al.,* 2016). Batista *et al.* (2013) reported that lipid biosynthesis is enhanced under nitrogen-limited conditions. Whey powder supports the growth of microalgae due to its main composition of lactose, which is a perfect source of carbon for microalgae, and carbon also increases lipid storage in microalgae (Chen *et al.,* 2024). Some microalgae groups can also be easily produced and have a fatty acid composition as MUFA, SFA, etc. for biodiesel, so whey powder was investigated as a potential source in microalgae cultivation for biodiesel production (Girard *et al.*, 2014).

Studies on the mechanism of microalgal protein production continue, and abiotic factors, such as carbon dioxide and light, are necessary requirements for protein production. However, some studies have shown that nitrogen is a key component of the cell structure and functional processes of microalgae, as it is a key element in proteins, amino acids, nucleic acids, enzymes, and photosynthetic pigments (Illman *et al.,* 2000). In addition, whey has been reported to positively affect protein production (Wang *et al.,* 2018), but studies on this subject are limited. Recently, applications of microalgae ranging from food production to nutritional supplements have increased interest in their use as a sustainable protein source (Tork *et al.*, 2022; Jalili *et al.*, 2024).

Microalgal pigments, including phycocyanin, phycoerythrin, astaxanthin, and carotene, are commercially utilized as food additives and colorants, and serve as valuable resources in fish and various food processing industries. In numerous

markets, microalgal carotenoids compete with their synthetic counterparts. Synthetic forms, although less expensive, may cause allergic reactions, including edema and chronic urticarial, etc. In contrast, microalgal carotenoids provide natural isomers in their natural ratios, which are healthier (Spolaore *et al.,* 2006; Minhas *et al.*, 2016). The most important pigments, which are commercially produced from microalgae, are beta-carotene (*Dunaliella salina*), phycocyanin (*Spirulina* sp.), astaxanthin (*Haematococcus pluvialis*), lutein (*Muriellopsis* sp.) and phycoerythrin (*Porphyridium cruentum*) (Koksal, 2008). One of these important pigment groups, carotenoids, can be divided into two categories based on their chemical structures: (1) carotenes, β-carotene, and (2) xanthophylls and fucoxanthin (Fu *et al.*, 2015). Phycocyanin and phycoerythrin are currently used as naturally pigmented fluorescent proteins with versatile applications (Mishra *et al.*, 2012). Astaxanthin (3,3'dihydroxy-β, β-carotene-4,4'-dione), a carotenoid, is a pigment in *H. pluvialis* (Jin *et al.*, 2024) and is commonly responsible for salmon colour in nature (Bustos-Garza *et al.,* 2013). Astaxanthin protects cell membranes and tissues against lipid peroxidation and oxidative stress. It has been reported that astaxanthin production increases and lipid peroxidation decreases in culture media containing 15, 25, 30, 35, and 40% whey permeate (Guerin *et al.,* 2003; Zimermann *et al.,* 2020).

The growth of microalgae is influenced by environmental factors such as nitrogen (N) and phosphorus (P) ratios, light availability, and carbon concentration. Both organic and inorganic sources can serve as carbon inputs in microalgal cultures (Girard *et al.,* 2014; Fulbright *et al*., 2016). Thus, Abreu *et al*. (2012) employed mixotrophic cultivation using nutrient media enriched with cheese whey powder solution supplemented with a mixture of glucose and galactose; however, in the present study, 3% inorganic carbon was added together with whey powder.

Whey powder is a complex compound consisting of protein, lactose, lipids and minerals. However, lactose is the main component of whey powder (around 70%). Whey powder also contains significant amounts of vitamins C, B1, B2 and folic acid. With this feature, whey powder provides a sufficient nutrient medium for the increase in microalgae biomass and the development of biochemical structures. Indeed, many studies have been conducted to understand the effects of whey powder on microalgae growth and metabolic composition (Abreu *et al.,* 2012; Girard *et al.,* 2014; Tsolcha *et al.,* 2015; Wang *et al.,* 2018). This study aimed to investigate the effect of whey powder with different concentrations on the biomass and lipid, protein, and pigment contents of *Scenedesmus* sp. and *H. pluvialis*. In this way, it was evaluated whether WP is useful or not as a supplement in the medium for microalgae culture.

Material and methods

Culture conditions of H. pluvialis and Scenedesmus sp.

H. pluvialis was provided by Çukurova University, Faculty of Fisheries, Turkey. *Scenedesmus sp.* was isolated from a natural freshwater resource (Atatürk University, Faculty of Fisheries, Pond) in

Erzurum, Turkey. Whey powder (WP) (moisture 0.87%, lipid 1%, protein 6.6%, ash 5.5%-8.6%, lactose 82%, pH 6.5, salt 1.98%, color light yellow) was purchased from Cici Dairy Industry Trade Inc., Turkey.

The microalgae were grown in 10 mL tubes, and then they were transferred to 100 mL and 250 mL Erlenmeyer flasks. The cultures were maintained at 25 ºC in a 43.15 µmol m^2s^{-1} lighting and 110 rpm shaking incubator (JRS Lab 32 brand) under a 16:8-hour day/night photoperiod. For intensive production, they were used in containers with lids in the algal unit with 3 L glass. Modified 3N-BBM+V was used as the nutrient medium. It is contained:
25 gL^{-1} $NaNO_3$, 2.5 gL^{-1} $CaCl_2.2H_2O$, 7.5 gL^{-1} K_2HPO_4, 7.5 gL^{-1} $MgSO_4.7H_2O$, 0.75 gL^{-1} Na2EDTA, 17.5 gL^{-1} $KH_2PO_3.3H_2O$, 2.5 gL^{-1} NaCl and supply of essential micronutrients ($FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $ZnCl_2$, $CoCl_2.6H_2O$ and $Na_2MoO_4.2H_2O$) (Anonymous, 2022).

Experimental design

The experimental design was set up with four groups and all groups had three replicates. The control group (C) contained only Bold Basal Medium with 3-fold nitrogen and vitamins (3N-BBM+V) without whey powder. The second experimental group was formulated as 3N-BBM+V supplemented with 5 g L^{-1} whey powder (W5), the third group as 3N-BBM+V supplemented with 10 g L^{-1} whey powder (W10), and the fourth group as 3N-BBM+V supplemented with 15 g L^{-1} whey powder (W15). All culture media used in the trial were first autoclaved at 121°C for 15 min and then vitamins were added. In this study, first both *H. pluvialis* and *Scenedesmus* sp. were taken from pre-cultured and then both species were cultured more intensively to investigate biomass, biochemical content and pigment concentration. All of the groups were set up with both species cultured in 3-L glass-covered containers with an initial biomass concentration of 0.08 g L^{-1}. Optimum culture conditions were carefully determined for each species, and all experimental treatments were conducted in triplicate. The experiment was completed, and the biomass of the species began to decline. *H. pluvialis* exhibited a biomass reduction on the 12th day, while *Scenedesmus* sp. showed a similar decrease on the 7th day, which was determined to be the lysis phase.

Determination of microalgal growth

The biomass of *H. pluvialis* (a) and *Scenedesmus* sp. (b) was calculated every day during the growth phases according to Li *et al.* (2021). Each day, 15 ml of sample from each trial was centrifuged at 4500 rpm for 5 min, then the supernatant was discarded and the microalgae biomass concentration was measured at the optical density of 680 nm using a UV spectrophotometer (Beckman). Microalgal biomass was calculated using the following formula:

Biomass (g/L) = 0.668 x (OD_t - OD_0) (R^2=0.9998) (a)

Biomass (g/L) = 0.482 x (OD_t - OD_0) (R^2=0.9996) (b)

Where, OD_t is the absorbance of algae at the sampling time, and OD_0 is the absorbance of the algae-free medium.

Pigments analysis

Chlorophyll-a concentration was determined according to Strickland and Parsons (Strickland and Parson, 1972). Carotenoid analysis was performed using the following formula after subtracting the OD750 value from the OD480. The microalgae sample, which was taken 1 ml for carotenoid analysis, was centrifuged at 13400 rpm and 4°C for 5 min, the liquid part was discarded, 1.5 ml of methanol was added to the sample and kept at 40ºC for 1 h. Then, the samples were centrifuged, and the optical density at 480 nm was read in the spectrophotometer. In the calculation of this analysis result, turbidity correction was performed by taking the difference of the optical density value at 750 nm from the optical density values obtained at 480 nm wavelengths (Ritchie, 2006; Kim et al., 2014). Phycocyanin concentration (C-Phycocyanin amount) (mg mL-1) was found using optical density values at 652 and 620 nm according to Moraes *et al*. (2011). The phycoerythrin concentration (mg mL-1) was determined from the optical density values at 562 and 615 nm according to Bennett and Bogorad (1973).

In this study, microalgae were subjected to light stress during the stationary phase (These phases were recorded on day 7th for *H. pluvialis* and on day 4th for *Scenedesmus sp.*), after which astaxanthin content was analyzed. Astaxanthin extraction was carried out according to Sarada *et al.* (2006). For this purpose, an astaxanthin standard (Sigma-Aldrich, St. Louis, MO, USA) was prepared and the amounts were determined using the calibration curve obtained using this standard. The samples were kept at -20°C until quantification by HPLC.

Total lipid analysis

Total lipid, fatty acid, and protein compositions were analyzed at the end of the stationary phase (These phases were recorded on day 12th for *H. pluvialis* and on day 7th for *Scenedesmus sp.*). Biomass was calculated to determine the total amount of lipid concentration (Chatzifragkou *et al.,* 2011). The lipid productivity was determined by the weight of lipid produced per liter of broth (g), and lipid yields were calculated with dry weight (Enshaeieh *et al.,* 2014). Fatty acid methyl esters analysis was carried out using the protocol of Metcalfe and Schmitz (1961), and a fatty acid methyl ester mix (Supelco, FAME-mix, 4–7801, Bellefonte, PA, USA) was used as standard. The fatty acid methyl esters were determined using a GC/FID (Agilent Technologies 6890N) system.

Total protein analysis

The protein was quantified using the Kjeldahl method. The wavelength was measured at 750 nm (Moein *et al.,* 2015). The curve standard was determined by BSA and the algal protein content was above the BSA standard curve (Lowry *et al.,*1951).

Statistical analysis

This study assessed the impact of whey powder application on the biomass, biochemical, and pigment composition of *H. pluvialis* and *Scenedesmus* sp. using one-way analysis of variance (ANOVA) by IBM SPSS 20 software. The evaluation included biomass, chlorophyll-a, astaxanthin, carotenoid, fucoxanthin, and

phycoerythrin levels, as well as protein, lipid, and fatty acid concentrations in response to varying whey powder concentrations. Mean differences were identified using Duncan's multiple range test at $p<0.05$. Additionally, the influence of whey powder (WP) supplementation on the biomass and chlorophyll-a of *H. pluvialis* and *Scenedesmus* sp. over different time periods (0 and 7 days), and at various WP concentrations (0, 5, 10, and 15 g L^{-1}) in different medium formulations was analysed using variance and correlation analysis with the MINITAB 15 software program.

Results

H. pluvialis and Scenedesmus sp. growth under mixotrophic conditions

Mixotrophic conditions (3N-BBM+V + WP) for *H. pluvialis* induced rapid growth stimulation until the 5th day and then showed a somewhat stationary growth phase for *H. pluvialis*, after which it fluctuated until the 11th day. Under mixotrophic conditions, *Scenedesmus* sp. exhibited rapid growth until the 4th day, after which fluctuations in biomass were observed through the end of the experiment on day 7. By the 7th day, a decline in growth rate was evident across all experimental groups. It can be a reason that *Scenedesmus* sp. passes to the stationary phase faster than *H. pluvialis* is that the lactose and nitrogen in the nutrient medium trigger *Scenedesmus* sp. to multiply in a short time, thus causing the rapid decrease of nutrients in the medium.

In this study, biomass production in both H. pluvialis and Scenedesmus sp. increased with rising whey powder concentrations, and these increases were statistically significant ($p<0.05$). The mean biomass of *H. pluvialis* and *Scenedesmus* sp. was approximately two times higher compared to the control group (0.45 gL^{-1} for *H. pluvialis* and 0.23 gL^{-1} for *Scenedesmus* sp.) (Fig. 1). *H. pluvialis* cultures reached the highest biomass yield (0.88 gL^{-1}) in the WP 10 groups, and *Scenedesmus* sp. cultures in the WP15 group (0.45 gL^{-1}). It is thought that using lactose, as well as a 3-fold enrichment of N in BBM medium, had a positive effect on the biomass increase of both species.

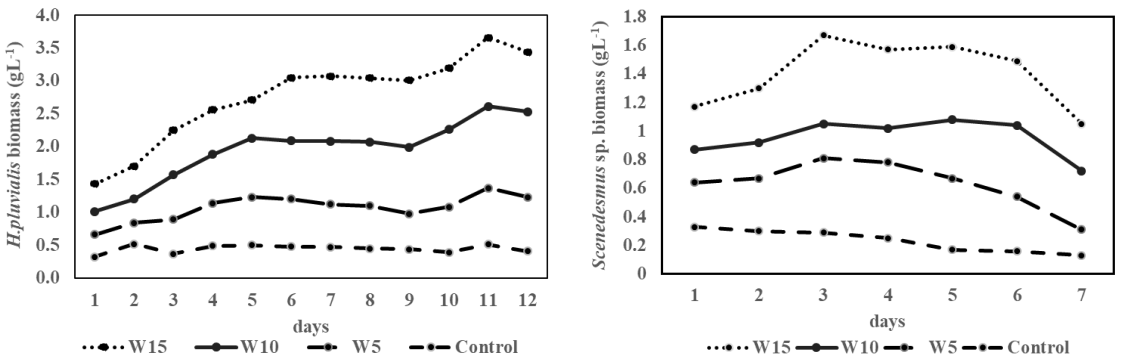

Figure 1: The biomass and chlorophyll-a values in *Haematococcus pluvialis* and *Scenedesmus* sp. vary depending on the group and the day.

Pigment production of H. pluvialis and Scenedesmus sp.

Chlorophyll-a (Chl-a), astaxanthin, carotenoid, and phycocyanin concentrations of *H. pluvialis* and *Scenedesmus* sp. under different concentrations of whey powder enrichment were found statistically significantly different (p<0.05). The biomass of *H. pluvialis* and *Scenedesmus* sp. showed a rapid increase under mixotrophic conditions during the log phase (Fig. 2).

Whey powder has been shown to support *Scenedesmus* sp. growth but has little effect on *H. pluvialis* growth based on Chl-a concentration, and the mean Chl-a of *H. pluvialis* groups were found to be 1.60 gL^{-1} (Control), 1.35 gL^{-1} (WP5), 1.14 gL^{-1} (WP10), and 1.51 gL^{-1} (WP15), respectively, while the mean Chl-a values were calculated for *Scenedesmus* sp. groups to be 4.48 gL^{-1} (Control), 6.91 gL^{-1} (WP5), 5.62 gL^{-1} (WP10) and 6.03 gL^{-1} (WP15), respectively.

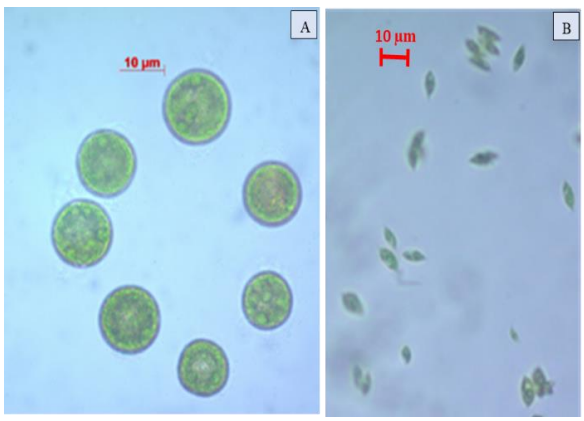

Figure 2: Microscope images of *Haematococcus pluvialis* and *Scenedesmus* sp. under mixotrophic conditions. (A: Image of *H. Pluvialis* during biomass production and B: Image of *Scenedesmus* sp. during biomass, 200X and 400X, Orijinal).

In the present study, carotenoid and astaxanthin production for both species had the highest values under mixotrophic conditions. The highest phycocyanin of *H. pluvialis* and *Scenedesmus* sp. was determined in WP 15. Carotenoid was found to have the highest value in the W10 group of *H. pluvialis* and W5 group of *Scenedesmus* sp. under mixotrophic conditions (Fig. 3). In this study, carotenoid production was found to be higher than phycocyanin production. Since no nutrients were added during the experiment, nutrients such as nitrogen and phosphorus decreased in the medium, which is thought to have caused a decrease in phycocyanin production but an increase in carotenoid production. The other possibility is that the increase in oxygen levels as a result of photosynthesis may have caused cellular stress, which may have increased carotenoid production.

Although astaxanthin production is well known in *H. pluvialis*, only a limited number of studies have reported astaxanthin production in *Scenedesmus* (Barouh *et al.,* 2024). Astaxanthin biosynthesis in *H. pluvialis* was primarily influenced by light intensity rather than WP supplementation. Light distribution and NaCl-induced stress likely accounted for the higher astaxanthin content in the control (21.31 mg/kg) and WP5 (22.30 mg/kg) groups than WP 10 (10.85 mg/kg) and WP15 (10.3 mg/kg). In *Scenedesmus* sp., astaxanthin accumulation (9.86 mg/kg in control, 12.07 mg/kg in WP5, 14.44 mg/kg in W10, and 18.21 mg/kg in W15) was enhanced under WP supplementation (Fig. 3).

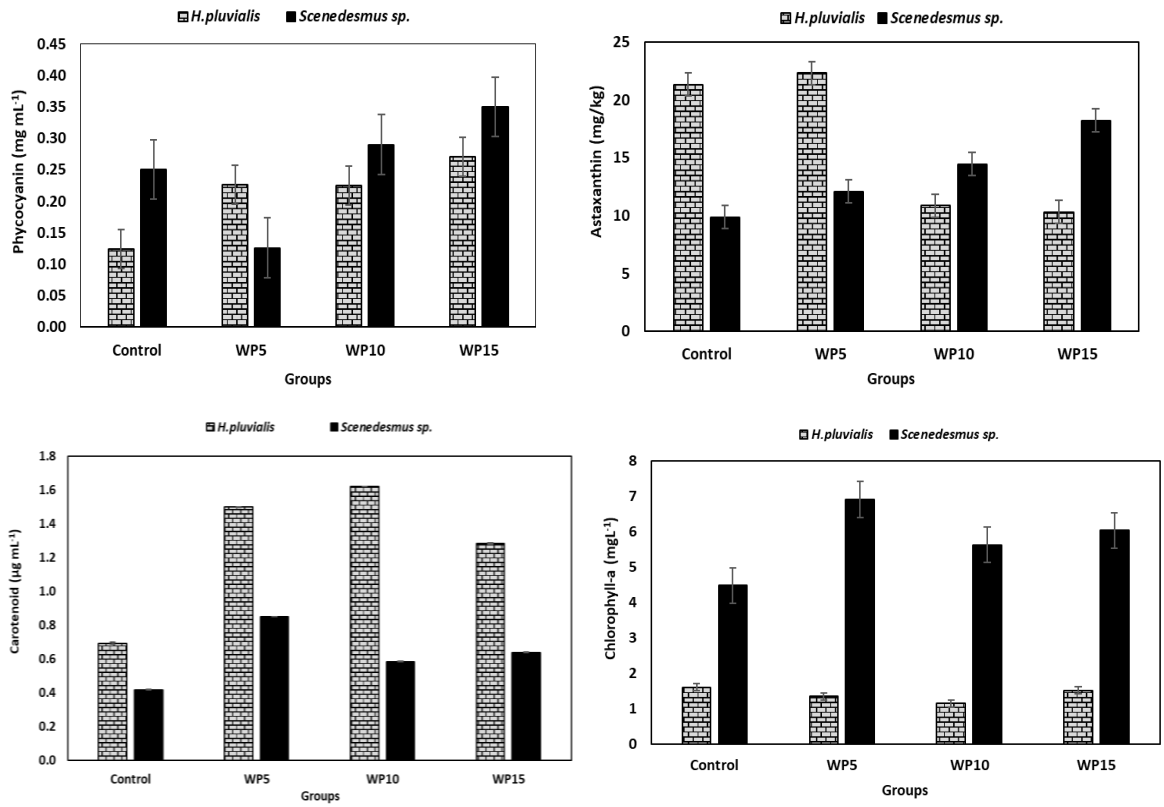

Figure 3: Changes in carotenoid, chlorophyll-a, phycocyanin, and astaxanthin yield of *Haematococcus pluvialis* and *Scenedesmus* sp. depend on the groups.

Lipid and protein production of H. pluvialis and Scenedesmus sp.

In this study, the lipid and protein productivity of *H. pluvialis* and *Scenedesmus* sp. under different whey enrichment concentrations of the nutrient medium were determined, and these parameters were found to be statistically significantly (p<0.05). The highest total lipid productivity was calculated for both *H. pluvialis* and *Scenedesmus* sp. as 43% (WP5) and 66% (WP10) w/w dry weight, respectively. The change in the total lipid value of *H. pluvialis* was observed to change depending on the whey powder treatment groups. The control group had 9% w/w dry weight, WP10 and WP15 groups had 25% w/w dry weight. The change in the total lipid productivity of *Scenedesmus* sp. was determined in the control and WP 5 groups as 21% w/w dry weight, WP10 group as 66% w/w dry weight and WP15 groups as 4% w/w dry weight. Lipid production for *H. pluvialis* and *Scenedesmus* sp. was observed to be better under mixotrophic conditions compared to autotrophic conditions. The highest protein content was determined in the W10 group at 20.94 mg mL^{-1} for *H. pluvialis* and in the W15 group as 44.94 mg mL^{-1} for *Scenedesmus* sp. under mixotrophic conditions (Fig. 4).

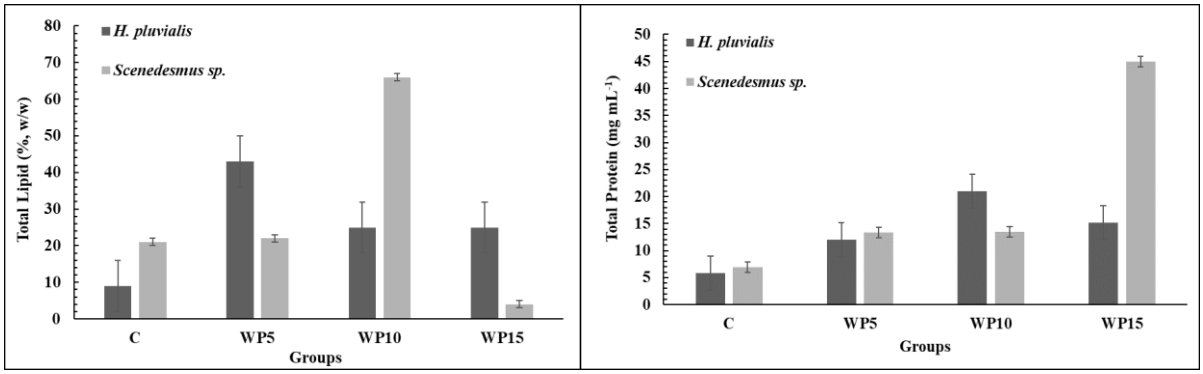

Figure 4: The change in total lipid and protein productivity is dependent on both *Haematococcus pluvialis* and *Scenedesmus* sp. groups.

The combined use of lactose in WP and nitrogen-enriched BBM medium provided both the organic carbon source required for biomass and the nitrogen source required for protein production in both species. The highest mean values of total lipid productivity for the fatty acid composition of both species were determined for saturated fatty acids (p<0.05). Cavallini *et al.* (2024) reported that the most important factor in the production of lipid and fatty acids by microalgae is mixotrophic culture conditions, and it is known that the saturated fatty acid (SFA) content is higher compared to PUFA in both *H. pluvialis* and *Scenedesmus* sp. (Both of them are in the group Chlorophyta) under both autotrophic and mixotrophic culture conditions, unlike Cyanobacteria. However, in this study, an increase in PUFA values was observed in experiments with WP concentrations of 5 gL^{-1} for *H. pluvialis* and WP concentrations of 10 gL^{-1} for *Scenedesmus* sp.. The fatty acid composition of *H. pluvialis* and *Scenedesmus* sp. under mixotrophic conditions showed that the dominant accumulated fatty acids were palmitic (C16:0) (37.81% and 41.88%). However, the highest value of C16:0 in *H. pluvialis* was calculated in the control group as 39.61%, whereas the highest value in *Scenedesmus* sp. was found in the W15 group (41.88%). The second dominant fatty acid composition of *H. pluvialis* and *Scenedesmus* sp. under mixotrophic conditions was found to be linoleic (C18:2n6) (21.81% and 26.79%). In addition, the PUFA concentration was higher than that of SFA in the WP10 group in *Scenedesmus* sp. (Tables 1 and 2).

Table 1: Fatty acid composition of *Haematococcus pluvialis* (%).

Fatty acids	Control	WP5	WP10	WP15
C14:0 (Myristic Acid)	0.48±0.21	3.44±1.33	2.64±0.15	2.9±1.06
C16:0 (Palmitic Acid)	39.61±0.72	37.74±2.4	37.81±1.23	35.34±0.34
C18:0 (Stearic Acid)	9.92±1.33	10.63±1.16	13.21±1.81	8.91±0.28
∑SFA**	50.01^{B*}	51.81^{B}	53.66^{A}	47.15^{C}
C16:1 (Palmitoleic Acid)	1.41±0.13	4.31±1.17	2.63±0.89	1.41±0.12
C18:1n9 (Elaidic Acid)	15.22±1.71	14.46±0.98	7.725±0.51	13.63±1.60
∑MUFA**	16.63^{B}	18.77^{A}	10.36^{C}	15.04^{B}
C18:2n6 (Linoleic Acid)	$14.88±2.22^{Ac}$	$18.74±4.84^{Ab}$	$21.20±0.52^{Aa}$	$21.81±1.70^{Aa}$
Fatty acids	Control	WP5	WP10	WP15
C18:3n6 (Linolenic Acid)	$12.85±2.94^{Ab}$	$6.77±1.03^{Bc}$	$12.55±1.34^{Bb}$	$13.82±2.39^{Aa}$
C20:5n3 (Eikosapentaenoik Acid)	$3.65±0.75^{Aa}$	$3.91±0.89^{Aa}$	$2.26±0.29^{Bb}$	$2.19±1.17^{Ab}$
∑PUFA**	31.38^{B}	29.42^{C}	36.01^{A}	35.63^{A}
∑PUFA/∑SFA	0.63^{B}	0.57^{B}	0.67^{B}	0.76^{B}
∑PUFA/∑MUFA	1.89^{C}	1.57^{C}	3.58^{A}	2.37^{A}
∑n3	3.65^{A}	3.91^{A}	2.26^{B}	2.19^{B}
∑n6	27.73^{B}	25.51^{B}	33.75^{A}	35.63^{A}
∑n3/∑n6	0.13^{A}	0.15^{A}	0.07^{B}	0.06^{B}
∑n6/∑n3	7.60^{B}	6.52^{B}	14.93^{A}	16.27^{A}

*A,B,C: Capital letters show the difference between groups of the same species, and the difference between groups with different capital letters in the same row is statistically significant (p<0.05).
** MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids

Table 2: Fatty acid composition of *Scenedesmus* sp. (%).

Fatty acids	Control	WP5	WP10	WP15
C14:0 (Myristic Acid)	16.64±1.92	10.72±3.65	11.08±1.91	11.96±7.98
C16:0 (Palmitic Acid)	30.85±1.68	40.13±1.54	26.98±0.27	41.98±0.86
C18:0 (Stearic Acid)	3.44±0.49	6.28±0.84	1.27±0.67	4.62±2.14
∑SFA**	50.93^{B}	57.13^{A}	39.33^{C}	58.56^{A}
C16:1 (Palmitoleic Acid)	13.76±0.53	4.37±1.99	2.13±0.1	1.78±0.4
C18:1n9 (Elaidic Acid)	11.69±0.63	9.29±0.23	10.64±2.74	6.91±1.62
∑MUFA**	25.45^{A}	13.66^{B}	12.77^{B}	8.69^{C}
C18:2n6 (Linoleic Acid)	12.98±0.63	14.03±0.67	26.79±1.73	21.7±0.82
C18:3n6 (Linolenic Acid)	6.29±0.13	12.01±1.38	18.09±2.11	7.52±0.62
C20:5n3 (Eikosapentaenoik Acid)	4.37±0.40	3.19±0.99	3.04±0.37	2.77±0.38
∑PUFA**	23.63^{C}	29.23^{B}	47.92^{A}	31.99^{B}
∑PUFA/∑SFA	0.46^{C}	0.51^{B}	1.22^{A}	0.55^{B}
∑PUFA/∑MUFA	0.93^{C}	2.14^{B}	3.75^{A}	0.55^{C}
∑n3	4.37^{A}	3.19^{B}	3.04^{B}	2.77^{C}
∑n6	19.27^{C}	26.04^{B}	44.88^{A}	29.22^{B}
∑n3/∑n6	0.23^{A}	0.12^{B}	0.07^{B}	0.10^{B}
∑n6/∑n3	4.41^{C}	8.16^{B}	14.76^{A}	10.55^{B}

*A,B,C: Capital letters show the difference between groups of the same species, and the difference between groups with different capital letters in the same row is statistically significant (p<0.05).
** MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids

Discussion

Mixotrophic growing conditions doubled the biomass compared to photoautotrophic growth for both species, when supplemented with whey powder in the media. Specifically, the biomass of *H. pluvialis* increased by 1.39, 2.08, and 1.52 times in the W5, W10, and W15 groups, respectively, compared with the control. Similarly, *Scenedesmus* sp. biomass was 1.92, 1.65, and 2.28 times greater in the W5, W10, and W15 groups, respectively. On the other hand, Aydoğdu and Fakıoğlu (2022) reported the highest biomass for *H. pluvialis* (1.26±0.55 g/L) in the nutrient medium with 15 g/L whey powder supplementation, which was significantly higher than the values found in the current study. Abreu *et al.* (2012) also noted biomass increases in *Scenedesmus* and *Chlorella* with the addition of whey powder. Moreover, the addition of glucose to whey powder has been reported to quadruple biomass (Girard *et al.,* 2014; Tsolcha *et al.,* 2015; Wang *et al.,* 2018; Li *et al.,* 2021). This suggests that, due to differences in culture conditions, cell structure can be influenced by genetic properties, also affects biomass in studies conducted under the same species and culture conditions.

Consistent with these findings, the current study observed the highest chlorophyll-a content in *Scenedesmus* sp. under mixotrophic conditions, which is consistent with Abreu *et al.* (2012), who found the highest levels under similar conditions. Sodium bicarbonate was used as an inorganic carbon source to increase biomass productivity and total chlorophyll content of *Chlorella vulgaris* and reported good performance (Rahmati *et al.,* 2024).

Microalgae such as *Chlorella*, *Dunaliella*, *Nannochloropsis*, and *Scenedesmus* produce various light-harvesting carotenoids and can manage oxidative stress. Phycocyanin serves as an intracellular nitrogen source and can be utilized under prolonged nitrogen-limited conditions (Sun *et al.,* 2017). The present study observed the highest production of carotenoids and phycocyanin under mixotrophic conditions.

H. pluvialis is recognized as a major source of astaxanthin, whereas *Scenedesmus* sp. is primarily known for carotenoid yield. Natural astaxanthin is synthesized by *H. pluvialis*, reaching concentrations of 1.5%–3% of the dry weight under natural conditions (Guerin *et al.,* 2003). Astaxanthin production has been reported at 5 mg/g dry weight in *H. pluvialis*, whereas at 0.27 mg/g in *Scenedesmus* sp., and Debnath et al. (2024) and Aditi et al. (2025) reported that values of up to 0.57 mg g^{-1} were achieved under salt and light stress conditions. In this study, WP supplementation enhanced astaxanthin accumulation in *Scenedesmus* sp., while in *H. pluvialis* this effect was observed only at WP concentrations of 5 gL^{-1}, whereas concentrations of 10 g/L and above inhibited astaxanthin production in *H. pluvialis*.

This study indicates that *Scenedesmus* sp. can be effectively grown under mixotrophic conditions for lipid production, unlike the lipid productivity of *H. pluvialis* under similar conditions. Studies have shown that incorporating whey powder into *Scenedesmus* culture medium enhances lipid production.

However, low nutrient concentrations, such as nitrogen (N) and phosphorus (P), which are essential for lipid synthesis, may decrease algal growth rate, final biomass concentration, and ultimately biodiesel production. Although microalgal growth rate was limited in nitrogen-deficient media, whey powder has been identified as a suitable organic carbon source for cultivating microalgae for biodiesel production (Girard *et al.,* 2014). Similarly, Wang *et al.* (2018) noted that different trophic conditions significantly affect lipid accumulation, with cells cultured under heterotrophic and mixotrophic conditions exhibiting substantially higher lipid contents than those cultured under autotrophic conditions.

The total amount and relative proportion of fatty acids can be affected by nutritional and abiotic factors as well as nitrogen limitation (Spolaore *et al.,* 2006). In this study, the percentage of palmitic acid (C16:0) production was higher than that of the other fatty acid compositions. Similar results have been found in other studies, where whey powder was applied: fatty acid methyl esters (FAMEs) containing saturated (35.8%), C18:0, and C16:0 fatty acid compositions were 43% and 24%, respectively, and C16:0 fatty acid content was 22.1% (Girard *et al.,* 2014; Tripathi *et al.,* 2015; Tsolcha *et al.,* 2015; Wang *et al.,* 2018). The saturated fatty acid composition is important, as it is considered in biodiesel production. *Scenedesmus* is a species on which much research has been conducted on biodiesel productivity (Chini *et al.,* 2023; Cavallini *et al.,* 2024). In this study, mixotrophic conditions with whey powder caused an increase in the saturated and unsaturated fatty acid composition; in particular, palmitic and linoleic fatty acid accumulation was found to be higher than other fatty acids. Palmitic fatty acid is a single-bond saturated fatty acid and is used in the cosmetic industry in skin care products and perfumes due to its saponification properties. Linoleic fatty acid is a carboxylic acid with an 18-carbon chain and three double bonds. Especially α-linolenic acid is recommended as part of a diet to prevent diseases such as cardiovascular disease, cancer, and coronary heart failure.

This study investigated the rate of lipid accumulation in cultures enriched with whey powder (mixotrophic conditions) compared with the control group (phototrophic conditions without whey powder). The productivity of unsaturated fatty acids was on average 3.5 times higher under mixotrophic conditions than under phototrophic conditions in *Scenedesmus* sp.. Although the content of PUFA (C18:2n6, C18:3n6, and C20:5n3) was lower than expected, an increase was observed in the samples treated with whey powder. The amount of PUFA and ω6/ω3 ratio varied significantly depending on the variety of microalgae species and cultivation conditions. For instance, the lipid content and quality of *Chlorella* differed notably, affecting the potential nutritional benefits of consuming commercial biomass, with ω6/ω3 ratios being lower (2.5) in photo-autotrophic mode, whereas in mixotrophic mode, they ranged from 1.3 to 8.9 in commercial biomass (Riano *et al.,* 2016). *Scenedesmus* sp. is recognized for its high SFA content, which makes it suitable for biodiesel

production. Culture condition parameters, such as temperature, lighting period, light intensity, and pH, were examined, particularly focusing on suspension and biofilm production methods for *Scenedesmus* sp. Recent studies have explored the possibility of consuming *Scenedesmus* spp. as food (Rai and Grupta, 2017). In fact, this study is expected to contribute to the evaluation of *Scenedesmus* sp. as a potential food source due to its PUFA content. Microalgae grown in a nitrogen-rich medium exhibited high protein concentration. Abreu *et al.* (2012) and Wang *et al.* (2018) have indicated that *C. pyrenoidosa* has a high protein content under mixotrophic conditions containing exogenous sugars such as glucose, galactose, mannose, fructose, sucrose, and lactose. Similarly, this study showed that the lactose contained in whey powder and inorganic carbon applications were effective in protein production for both species, and the highest protein content was determined in the W10 group as 20.94 mg mL^{-1} for *H. pluvialis*, and in the W15 group as 44.94 mg mL^{-1} for *Scenedesmus* sp.. This is consistent with the findings of El-Sheekh *et al.* (2004) and Becker (2007) on the effects of nitrogen sources on the growth and biochemical composition of *Scenedesmus* sp.

Many microalgae have protein content, accounting for 30-60% of their dry weight, so recently, extracts of *Scenedesmus*, *Chlorella*, and *Spirulina* have been incorporated as food additives in yogurt and cheese production, as well as microalgae powder, because of their functional food properties (Salah *et al.*, 2023; Amiri *et al.*, 2024; Bagheri *et al.*, 2024; Jalili *et al.*, 2024; Scarponi *et al.*, 2024). Moreover, *Arthrospira platensis* improves the general health and stress tolerance of Persian Sturgeon fry and their survival rate during artificial breeding (Akhoundian *et al.*, 2025). This study demonstrated that functional food properties, in terms of protein content, can be applied to both *H. pluvialis* and *Scenedesmus* sp.

Conclusion

As a conclusion, the present study demonstrates that the supplementation of the culture medium with whey powder significantly enhances both biomass production and biochemical composition. Whey powder is cost-effective with a sales price of 2.62 €/kg in Türkiye, raw material and rich in essential growth elements as a supplementary, so it can useful for microalgae culture media. Moreover, *H. pluvialis* and *Scenedesmus* sp. cultivated with whey powder supplementation demonstrated significant potential for nutraceutical and pharmaceutical applications due to their high biomass productivity and favourable pigment, protein, lipid, antioxidant, long-chain polyunsaturated fatty acid, and α-linoleic acid contents. Future studies should examine different species or culture media, and further investigation of fatty acid profiles and protein productivity using molecular approaches is recommended.

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Conflicts of interest

There are no conflicts of interest regarding this research for any of the authors.

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