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RESEARCH ARTICLE

Investigation of the effects of whey powder on *Haematococcus pluvialis* cell growth kinetics

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ABSTRACT

This study was carried out to examine the effect on the growth of *Haematococcus pluvialis* using low-cost whey powder (WP). The *H. pluvialis* used in this study were from Çukurova University, Faculty of Fisheries, and 70% demineralized WP from Cici Dairy Industry Trade Inc. The experiment was conducted under laboratory conditions with 3 replications. During this experiment, cell numbers and biomass were analyzed every day. In addition, the specific growth rate of *H. pluvialis* was calculated according to the Monod Equivalence. The mean values of the cell number following the WP application were calculated according to the groups (C, W5, W10 and W15) as 763.34±419.62 cells/ml, 951.60±388.20 cells/ml, 1105.27±380.35 cells/ml and 978.63±411.07 cells/ml, respectively. The mean biomass value has been found the lowest in the control group (0.84±0.36 g/l), and the highest value in the W15 group (1.26±0.55 gl^{-1}). The mean specific growth rate was determined as 0.52±0.09 day^{-1} in the control group, 0.56±0.1 day^{-1} in the W5 group, 0.56±0.14 day^{-1} in the W10 group, and as 0.61±0,09 day^{-1} in the W15 group. In accordance with the data obtained, both the biomass and the specific growth rate of *H. pluvialis* were observed to increase in the W15 group. The use of WP as a nutrient medium is recommended due to its low-cost as well as increasing biomass.

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Introduction

Nowadays, as a result of the rapid population growth and the gradual decrease of limited natural resources, the danger of famine has become the biggest problem for countries. Food products of plant and animal origin are insufficient to meet the needs of people day by day. It also is used methods such as chemicals and/or genetic modification of the product to fill the food deficient. However, these methods adversely affect human health. For this reason, countries have turned to functional food research. One of the important researches of functional food is algae which meet the functional food demand. Algae are important in terms of their high nutritional content and contribution to human health. Sasa et al. (2020) reported that β-carotene, carotenoid and polyunsaturated fatty acids produced by algae have positive effects on human health and even reduce the risk of chronic diseases when used directly or added as food additives (Aslanbay Güler & İmamoğlu, 2021).

One of the main factors affecting the growth parameter of microalgae is the nutrient concentration in the medium. Nitrogen (N) and phosphorus (P) are two macronutrients of medium concentration, and they play an important role in cell metabolism as many biochemical processes. Nitrogen is mainly used to form proteins, amino acids, and nucleic acids, while phosphorus is mostly a component of nucleic acids and phospholipids (Bougaran et al., 2010).

The microalgae growth rate is limited by N or P. However, the N:P ratio for maximum growth may vary from species to species. In microalgae growth theory has been assumed that multiple nutrients limited growth, and it has been experimentally proven: (1) The relationship between external nutrient concentration and uptake rate is expressed by the Michaelis-Menten Kinetic Model. (2) The relationship between a single limiting nutrient and growth rate also achieves concentration saturation. Combining these relationships, including a growth model with a single limiting nutrient, was tested in both stable and variable conditions of light and temperature conditions. (3) Regarding multiple limiting nutrients, Liebig's law of minimum has been shown to hold. Microalgae's growth is dependent only on the intracellular concentration of the most limiting nutrient (Klausmeier et al., 2004).

Microalgal growth kinetics are expressed with Monod and Droop models only when nutrient concentration is taken into account (Aslanbay Güler & İmamoğlu, 2021). In batch culture systems, if only one of the nutrients is limited for growth, first of all, the nutrient ratio decreases rapidly and eventually microalgae growth stops, while in non-batch culture systems, growth is limited. Experimentally, the effect of limited substrate or nutrient is determined by the Monod equation. This model is mostly used in cases where the effect of a single nutrient in the culture medium or on conditions with a low concentration nutrient medium. Models depend on parameters, which are not always easily measurable or available, and as a result, mass flows, dynamics, and physiological variables often cannot be adequately detected. Traditional mass-based models (usually single nutrient, N or C) are generally relatively simple and operate using classical intake kinetic relationships. However, even these classical relationships are poorly characterized for many species or highly variable under different growing conditions (Aslanbay Güler & İmamoğlu, 2021).

Dairy products are included in the functional food group due to the scientific support for their positive effects on health. Whey powder is the wieldiest used commercially dairy product. Because drying the whey both extends the shelf life of the product and provides easy portability. Whey powder (WP) is defined as a substance containing high lactose, which is formed as a result of turning whey into powder form by subjecting it to a drying process in appropriate facilities. Today, WP is safely used as a food additive in baby foods, yogurts, candies, bakery products, meat products, soups, sauces and beverages (Yıldırım & Güzeler, 2013).

In general research with *H. pluvialis* has focused on biomass change and increasing astaxanthin pigment content depending on medium conditions (light, temperature, etc.) (Göksan, 2003; Eriştürk, 2005; Akın, 2005; İmamoğlu, 2005; Köksal, 2008; Çokcan, 2015; Giritli Yılmaz, 2019; Kalmaoğlu; 2020). This study aimed to examine the effect on the growth of *H. pluvialis* by using whey powder.

Material and Methods

Microalgae and Culture Conditions

The microalga *H. pluvialis* was provided by Çukurova University, Faculty of Fisheries Turkey. 70% demineralized 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) from Cici Dairy Industry Trade Inc. The research has started to be carried out in the Basic Sciences Laboratory of Atatürk University, Faculty of Fisheries, Experimental Research Unit. Initially, microalgae were grown in 10 ml tubes. The microalgae that developed in the tubes were first taken into 100 ml and then 250 ml Erlenmeyer flasks, and were grown at 25°C, in a 43.15 $\mu molm^{-2}s^{-}$ lighting and 110 rpm shaking incubator (JRS Lab 32 brand)

in a 16:8-hour daylight period. For intensive production of microalgae, they were taken into 3l glass containers with lids in the Algae Unit in the research unit.

In the experiment was used 3N-BBM+V nutrient medium modified as a nutrient medium. It is contained: 25 g/l $NaNO_3$, 2.5 g/l $CaCl_2.2H_2O$, 7.5 g/l K_2HPO_4, 7.5 g/l $MgSO_4.7H_2O$, 0.75 g/l Na_2EDTA, 17.5 g/l $KH_2PO_3.3H_2O$, 2.5 g/l 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 Procedure

In the experiment, 3N-BBM+V + 5 g WP, 3N-BBM+V +10 g WP and the 3N-BBM+V +15g WP was added to medium per liter. After the sterilization application, predetermined algae prepared with 3N-BBM+V medium were inoculated into this medium. The control group (C) and the added groups (W5, W10 and W15) were formed with 3 replications each (Bold, 1949; Bischoff & Bold, 1963). No nutrient medium was added to any group, including the control group, during the trial period. pH was controlled by the on-demand injection of carbon dioxide into the air stream entering the culture. The room temperature of the experimental environment was adjusted to be 25°C. The experiment was completed on the 14th day, which is the transition phase.

Analytic Methods

Determination of microalgal growth

Cell numbers were made daily to examine the development period of *H. pluvialis*. 3 ml of homogeneous microalgae sample was taken and Lugol solution was dropped in the counting chamber and left for the night. Then, cell counts were made under the Zeiss Primo Vert model invert microscope (200X, 400X) (Utermöhl, 1958; Anonymous, 2003).

$$Phytoplankton\ count\ \left(\frac{cells}{ml}\right) = \frac{CxAt}{AsxSxV} \quad (1)$$

In this equality;

C: Number of organism (cell),

At: Count cell bottom area (mm^2),

As: Microscope field of view (mm^2),

S: Number of counted areas (cell),

V: Sample volume precipitated (ml).

The biomass of *H. pluvialis* was calculated every day during the production phase. Biomass measurement was done with a spectrophotometer to measure optical density. 50 ml of the homogeneous sample was taken and centrifuged at 13400 rpm and 4°C for 5 minutes. Then, A supernatant was discarded. The supernatants were dried at 80°C for 24 h. Readings were made at a wavelength of 680 nm on a Shimadzu UVmini-1240 brand spectrophotometer. Microalgae biomass was calculated from the following formula (Kang et al., 2005).

$$Dry\ cell\ weight\ (g/l) = 0.668 \times A_{680} \quad (2)$$

Kinetic Modeling

The Monod equivalence model is used when only one nutrient is limiting in the microalgae growth research. This equivalence assumed that the temperature and light intensity are constant throughout the production (Bougaran et al., 2010).

$$Monod\ equivalence: \mu = \frac{\mu_{max}.C_s}{K_s + C_s} \quad (3)$$

where, μ, specific growth rate (day^{-1}); C_s, substrate concentration (mgl^{-1}); μ_{max}, maximum specific growth rate (day^{-1}); K_s, Monod saturation constant (mgl^{-1}).

Statistical Analyzes

The variation of *H. pluvialis* biomass, cell count, specific growth rate according to Monod equivalence, depending on groups and days was determined by One-Way (ANOVA) test using IBM SPSS 20. The significance of the differences was evaluated according to the DUNCAN test.

Results

Cells Number of H. pluvialis

The difference in the cell number of *H. pluvialis* according to the days and groups was found to be statistically significant ($p<0.05$) (Table 1). The mean values of the cell number according to the groups (C, W5, W10 and W15) were determined as 763.34±419.62 cells/ml, 951.60±388.20 cells/ml, 1105.27±380.35 cells/ml and 978.63±411.07 cells/ml, respectively. In this study, the number of cells started to increase from the 4th day, in all groups except the control group, the stationary phase started from the 6th day and a decrease was observed after the 12th day. In the control group, after reaching the highest cell count on the 8th and 9th days, a rapid decrease was detected (Figure 1).

Table 1. Change of *H. pluvialis* cell number (cells/ml) depend on groups and days (n=3; Mean±SD)

Day	C	W5	W10	W15
1	358.57 ±33.07^{Be}	391.86 ± 58.22^{ABe}	405.18 ±12.44^{Ae}	473.66 ±106.49^{ABe}
2	360.47 ±8.24^{Be}	499.34 ± 0.00^{ABe}	423.25 ±23.24^{Ae}	440.37 ±28.01^{ABe}
3	399.47 ±0.00^{Be}	391.86 ± 8.24^{ABe}	435.61±10.80^{Ae}	470.80 ±64.50^{ABe}
4	459.39 ±0.00^{Bd}	752.34 ± 38.53^{ABd}	959.68 ±79.96^{Ad}	786.58 ±51.07^{ABd}
5	1034.82 ±37.99^{Bb}	1293.52 ± 64.33^{ABb}	1314.45 ± 31.43^{Ab}	867.42 ±0.00^{ABb}
6	1078.57 ±0.00^{Bab}	1269.75 ±116.78^{ABab}	1372.47 ±30.06^{Aab}	1192.70 ±0.00^{ABab}
7	1065.25±42.74^{Bab}	1284.01±0.00^{ABab}	1284.01 ± 49.99^{Aab}	1308.74 ±70.84^{ABab}
8	1392.44 ±0.00^{Bab}	1315.40 ±0.00^{ABab}	1412.41 ±9.88^{Aab}	1406.71 ±0.00^{ABab}
9	1560.79 ±0.00^{Ba}	1520.84 ±12.44^{ABa}	1520.84 ±15.72^{Aa}	1412.41 ±0.00^{ABa}
10	1113.76±23.06^{Bab}	1209.82 ±34.59^{ABab}	1397.20 ±9.17^{Aab}	1426.68 ±2.85^{ABab}
11	771.36 ±65.71^{Bab}	1216.48 ±31.30^{ABab}	1216.48 ±13.18^{Aab}	1420.97 ±0.00^{ABab}
12	468.90 ±78.37^{Bc}	998.68 ±135.56^{ABc}	1386.73±29.65^{Ac}	1398.15 ±0.00^{ABc}
13	325.28 ±44.479^{Bd}	752.34 ±101.46^{ABd}	1158.46±25.36^{Ad}	696.22 ±0.00^{ABd}
14	297.70 ± 1.647^{Bd}	426.10 ± 116.58^{ABd}	1102.27±12.44^{Ad}	399.47 ±0.00^{ABd}

Note: *A, AB, B: Capital letters show the difference between groups on the same day and the difference between groups with different capital letters on the same line is statistically significant ($p<0.05$). a, ab, b, c, d, e: Lowercase letters indicate the difference between days in the same group, and the difference between days with different lowercase letters in the same column is statistically significant ($p<0.05$).

Table 2. Change of *H. pluvialis* biomass (gl^{-1}) depend on groups and days (n=3; Mean±SD)

Day	C	W5	W10	W15
1	0.57±0.015^{Bc*}	0.66± 0.044^{ABc}	0.46± 0.031^{ABc}	0.77± 0.017^{Ac}
2	0.56±0.051^{Bc}	0.64± 0.015^{ABc}	0.46± 0.000^{ABc}	0.83± 0.006^{Ac}
3	0.55±0.017^{Bc}	0.63± 0.010^{ABc}	0.47± 0.021^{ABc}	0.83± 0.010^{Ac}
4	0.53±0.000^{Bc}	0.63± 0.006^{ABc}	0.50± 0.030^{ABc}	0.83± 0.006^{Ac}
5	0.56±0.035^{Bc}	0.64± 0.012^{ABc}	0.64± 0.025^{ABc}	0.86± 0.023^{Ac}
6	0.56±0.026^{Bc}	0.64± 0.006^{ABc}	0.63± 0.023^{ABc}	0.86± 0.010^{Ac}
7	0.57±0.029^{Bc}	0.63± 0.029^{ABc}	0.65± 0.013^{ABc}	0.88± 0.012^{Ac}
8	0.57±0.058^{Bc}	0.67± 0.017^{ABc}	0.69± 0.035^{ABc}	0.87± 0.012^{Ac}
9	0.67±0.035^{Bb}	1.56± 0.196^{ABb}	1.93± 0.082^{ABb}	1.56± 0.297^{Ab}
10	1.27±0.058^{Bab}	1.47± 0.195^{ABab}	1.89± 0.553^{ABab}	1.98± 0.391^{Aab}
11	1.35±0.078^{Ba}	1.70± 0.088^{ABa}	2.01± 0.106^{ABa}	2.26± 0.067^{Aa}
12	1.43±0.006^{Ba}	1.66± 0.053^{ABa}	2.14± 0.134^{ABa}	2.32± 0.182^{Aa}
13	1.32±0.089^{Bb}	1.57± 0.553^{ABb}	1.45± 0.398^{ABb}	1.44± 0.349^{Ab}
14	1.26±0.076^{Bb}	1.37± 0.324^{ABb}	1.29± 0.159^{ABb}	1.34± 0.097^{Ab}

Note: *A, AB, B: Capital letters show the difference between groups on the same day and the difference between groups with different capital letters on the same line is statistically significant ($p<0.05$). a, ab, b, c, d, e: Lowercase letters indicate the difference between days in the same group, and the difference between days with different lowercase letters in the same column is statistically significant ($p<0.05$).

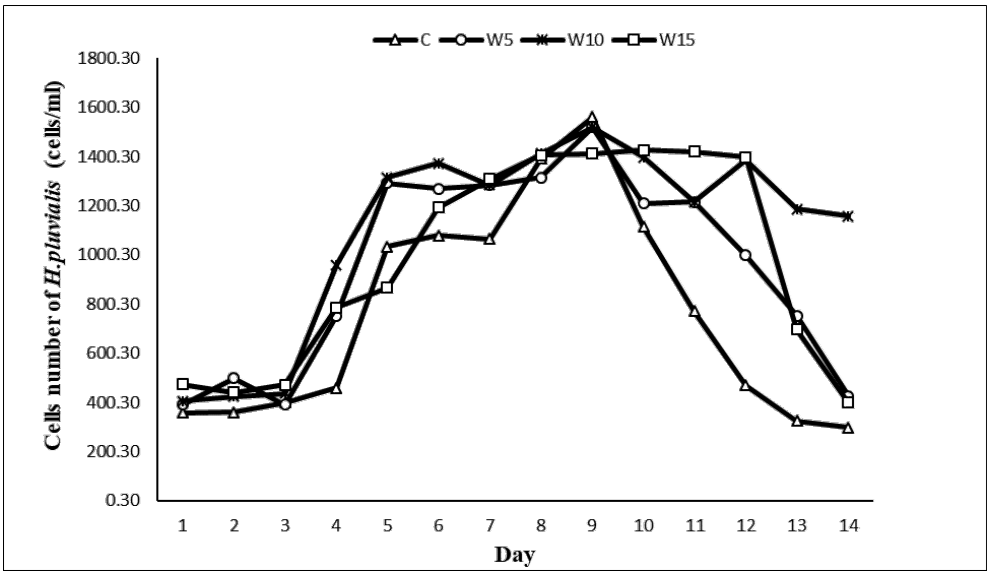

Figure 1. Change of *H. pluvialis* cell number (cells/ml) depend on days and groups

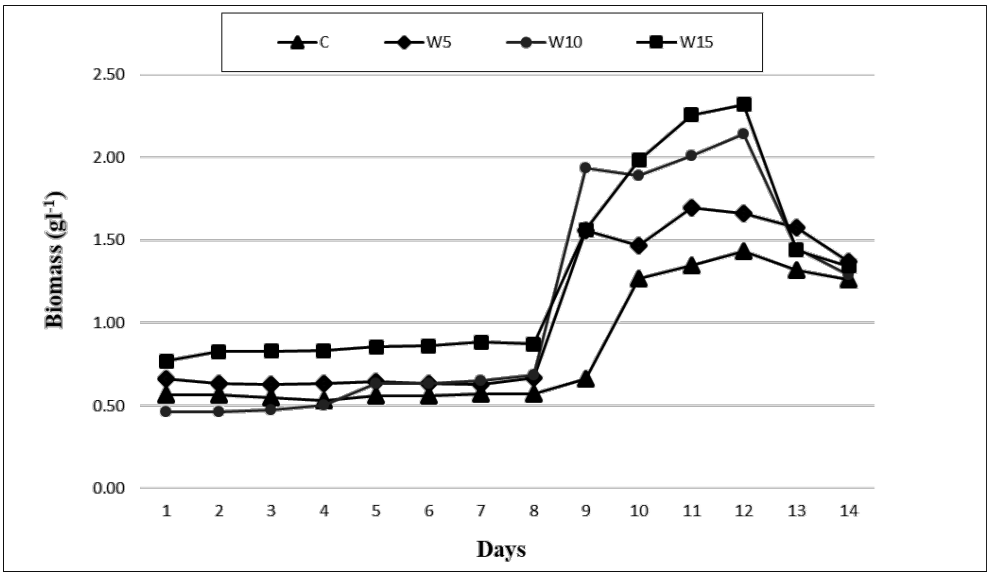

Figure 2. Change of *H. pluvialis* biomass (gl^{-1}) depend on days and groups

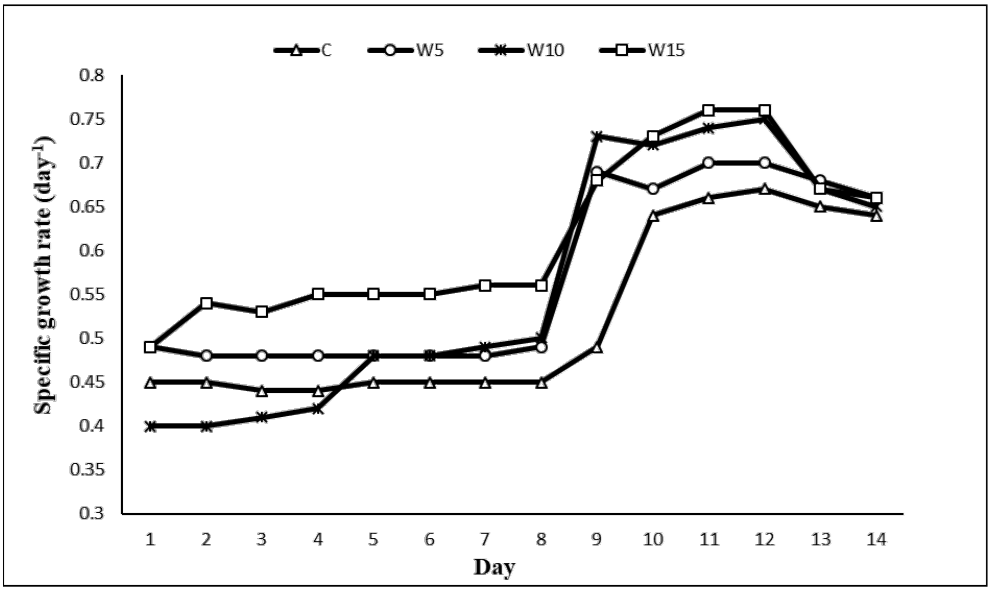

Figure 3. Change of *H. pluvialis* specific growth rate (day^{-1}) depend on days and groups

Table 3. Change of *H. pluvialis* specific growth rate (day^{-1}) depend on groups and days (n=3; Mean±SD)

Day	C	W5	W10	W15
1	0.45±0.00^{Bc*}	0.49±0.00^{ABc}	0.40±0.00^{ABc}	0.49±0.00^{Ac}
2	0.45±0.00^{Bc}	0.48±0.00^{ABc}	0.40±0.00^{ABc}	0.54±0.00^{Ac}
3	0.44±0.00^{Bc}	0.48±0.00^{ABc}	0.41±0.00^{ABc}	0.53±0.00^{Ac}
4	0.44±0.00^{Bc}	0.48±0.00^{ABc}	0.42±0.00^{ABc}	0.55±0.00^{Ac}
5	0.45±0.00^{Bc}	0.48±0.00^{ABc}	0.48±0.00^{ABc}	0.55±0.00^{Ac}
6	0.45±0.00^{Bc}	0.48±0.00^{ABc}	0.48±0.00^{ABc}	0.55±0.00^{Ac}
7	0.45±0.00^{Bc}	0.48±0.00^{ABc}	0.49±0.00^{ABc}	0.56±0.00^{Ac}
8	0.45±0.00^{Bc}	0.49±0.00^{ABc}	0.50±0.00^{ABc}	0.56±0.00^{Ac}
9	0.49±0.00^{Bb}	0.69±0.00^{ABb}	0.73±0.00^{ABb}	0.68±0.00^{Ab}
10	0.64±0.00^{Bab}	0.67±0.00^{ABab}	0.72±0.00^{ABab}	0.73±0.00^{Aab}
11	0.66±0.00^{Bab}	0.70±0.00^{ABab}	0.74±0.00^{ABab}	0.76±0.00^{Aab}
12	0.67±0.00^{Ba}	0.70±0.00^{ABa}	0.75±0.00^{ABa}	0.76±0.00^{Aa}
13	0.65±0.00^{Bab}	0.68±0.00^{ABab}	0.67±0.00^{ABab}	0.67±0.00^{Aab}
14	0.64±0.00^{Bb}	0.66±0.00^{ABb}	0.65±0.00^{ABb}	0.66±0.00^{Ab}

Note: *A, AB, B: Capital letters show the difference between groups on the same day and the difference between groups with different capital letters on the same line is statistically significant ($p<0.05$). a, ab, b, c, d, e: Lowercase letters indicate the difference between days in the same group, and the difference between days with different lowercase letters in the same column is statistically significant ($p<0.05$).

H. pluvialis Biomass

The difference in *H. pluvialis* biomass according to the day and groups was found to be statistically significant ($p<0.05$). The mean biomass value was determined the lowest in the control group (0.84±0.36 gl^{-1}), and the highest value in the W15 group (1.26±0.55 gl^{-1}) (Table 2). The highest biomass value was calculated on the 12th day in all groups except W5. Biomass increase started after the 4th day and reached the highest value on the 12th day (Figure 2).

Kinetic Modeling (Monod Equivalence)

Changes in specific growth rates were found to be significant ($p<0.05$) depending on between the groups and the days. It was calculated that the growth rate was the highest value (0.61 day^{-1}) in the W15 group. This group was followed by W5, W10 and control groups with an average of 0.57 days^{-1},0.56 days^{-1} and 0.52 days^{-1}, respectively (Table 3).

When the specific growth rate of the *H. pluvialis* was calculated by Monod equivalence, it was determined that the growth rate increased in all groups from the 4th day, similar to the change in biomass, and reached the maximum level on the 12th day (Figure 3).

During the experiment, the highest value of cell number in the W10 group has been found compared with the other groups. The lowest biomass has been calculated at 0.84±0.36 gl^{-1}in the control group, and the mean biomass value in the W5 and W10 groups was found 1.03±0.46 gl^{-1}and 1.09±0.64 gl^{-1}, respectively. The highest biomass value has been calculated as 1.26±0.55 gl^{-1} in the W15 group. The mean specific growth rate was determined as 0.52±0.09 day^{-1} in the control group, 0.56±0.1 day^{-1} in the W5 group, 0.56±0.14 day^{-1} in the W10 group, and 0.61±0.09 day^{-1} in the W15 group, respectively (Figure 4).

Discussion

Whey powder does not show the same effect in all microalgae species. According to Girard et al. (2014), *S. obliquus* reached the highest cellular concentrations (25±4×10^6 cells/ml); It has been reported that lactose supports the growth of *Scenedesmus* but does not change the cell number of *C. protothecoides*. In this study, it was determined that the cell number of *H. pluvialis* to which WP was added increased when compared to the control group.

The difference in cell numbers between the groups throughout the study was found to be statistically significant ($p<0.05$). The lowest cell number was calculated in the control group, and the highest cell number was calculated in the W10 group. In *Chlorella prenoidosa* culture, tofu whey was added to different concentrations (0, 20%, 40%, 60%, 80% and 100%) and its effect on cell growth was investigated. They found that the use of whey wastewater increased the number of cells more than

only the BBM medium, however, the cell number was 83.5×10^6 cells/ml in the use of only whey wastewater. They reported that whey is a suitable and inexpensive nutrient medium to increase cell number in microalgae cultures (Wang et al., 2018).

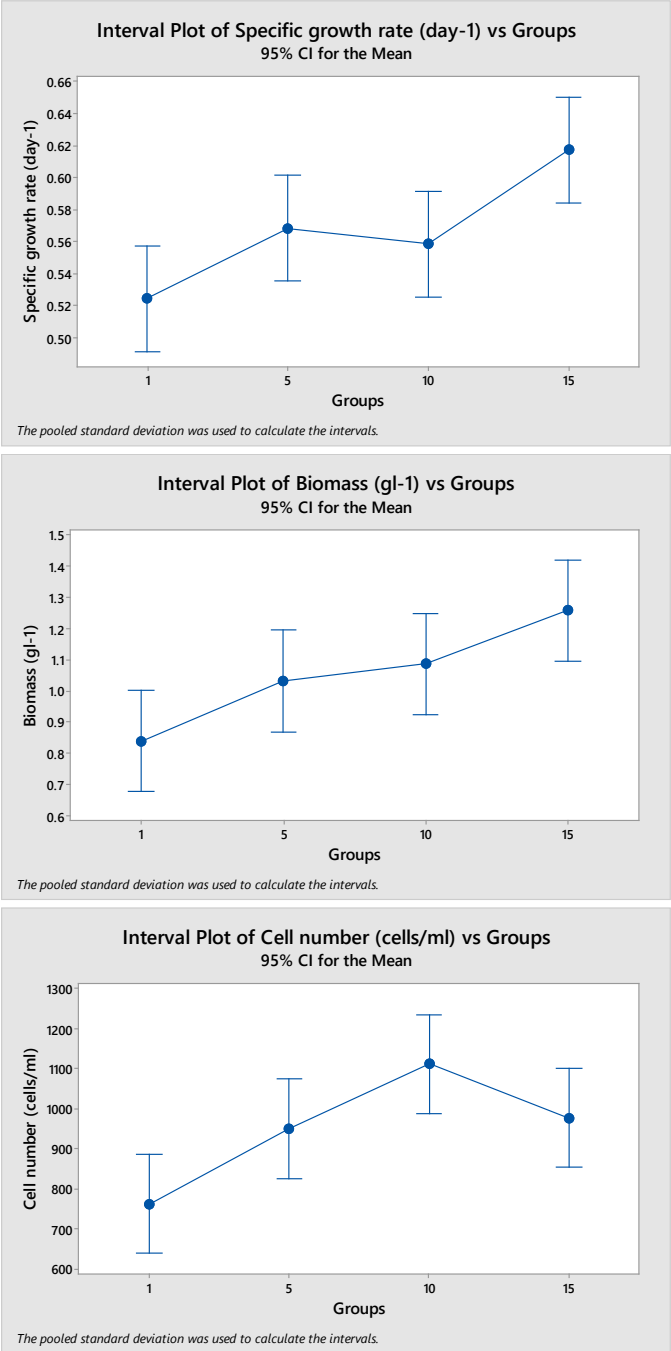

Figure 4. Change of *H. pluvialis* cells number (cells/ml), biomass (gl^{-1}) and specific growth rate (day^{-1}) depend on groups

It has been reported that in addition to the presence of nitrogen and phosphorus basic nutrients in microalgae biomass increase, the addition of inorganic and organic carbon increases photosynthesis (Girard et al., 2014). Studies conducted on some species of *Scenedesmus* and *Chlorella* in the Chlorophyta group have reported that the use of whey and/or powder increases the biomass more than using only the nutrient ratios suitable for the species (BBM, BG-11, etc.). However, in the same studies, the addition of glucose to the use of whey and/or whey powder increased the biomass 3-4 times, while the biomass of *C. prenoidosa* was 0.28 gl^{-1} only in BG-11 medium, its biomass was determined 4.76 gl^{-1} in the medium created by adding whey and glucose (Girard et al., 2014; Tsolcha et al., 2015; Wang et al., 2018).

As a carbon source, glucose and galactose were applied to the *Chlorella vulgaris* medium; They calculated the biomass value as 1.22 gl^{-1} only in WP application and as 2.24 gl^{-1} in only glucose + galactose application (Abreu et al., 2012). In this study, the highest biomass value was determined as 2.32± 0.18 gl^{-1} in the use of 15gl^{-1} whey powder. In the experiment we have done at different concentrations, it is predicted that the use of WP may increase biomass depending on the species.

The use of WP in microalgae media has a specific growth rate stimulating effect due to its high nutrient salts (N, P) content (Abreu et al., 2012). *Chlorella vulgaris*, which is also in the Chlorophyta group, was applied to the medium as a carbon source of whey powder, glucose and galactose. The specific growth rate was determined as 0.43 day^{-1} in the use of only whey powder and as 0.47 day^{-1} in the application of whey powder and glucose+galactose (Abreu et al., 2012). In this study, the highest specific growth rate was calculated as 0.76 day^{-1} in the use of 15 gl^{-1} whey powder. In studies with multiple limiting nutrients, the growth rate only depends on the microalgae intracellular concentration of the most limiting nutrient (Klausmeier et al., 2004).

Conclusion

Microalgae are used in many fields from the food industry to the pharmacy. The biggest problem in microalgae culture in these sectors is increasing the biomass of the selected species and supplying the lack of a cheap culture medium.

As a result, in this study, WP a by-product of the dairy industry was added to the culture medium for the development of *H. pluvialis* and at the end of the experiment, it was determined that WP had a positive effect on biomass. The addition of 15 gl^{-1} WP for *H. pluvialis* culture increased both the biomass and specific growth rate of *H. pluvialis*. The use of WP as a medium is recommended due to its low-cost as well as increasing biomass.

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Compliance With Ethical Standards

Authors' Contributions

Both authors have contributed equally to the paper. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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