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Determination of the effect of whey as a nutritional supplement in different growth medium regarding to its potential to biodiesel feedstock production

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ABSTRACT

Large-scale production of microalgae is a costly process because of high costs of microalgae feed, artificial lighting and operational costs. Whey (CW) is one of the agricultural waste materials which contains high amounts of protein and minerals and is considered as a feed source for *Chlorella vulgaris*. The objective of this research was to determine the effects of whey on biomass production of *Chlorella vulgaris*. *Chlorella vulgaris* was produced in four different growth medium of 1) Blue Green Medium(BG11) *Chlorella vulgaris* 2) Blue Green Medium(BG11) whey (CW) *Chlorella vulgaris* 3) Bold's Basal Medium (BMM) *Chlorella vulgaris* and 4) Tap water(TW) *Chlorella vulgaris*. After 21 days of experimentation, the highest number microalgae cells, biomass gain and lipid were observed in Bold's Basal Medium (BMM) *Chlorella vulgaris* growth medium containing as 79.7x10⁶ cell mL⁻¹, 10.14 g L⁻¹ and 20.7%, respectively. It is found that whey can be considered as a promising feed source for the production of *Chlorella vulgaris*.

Keywords:

Microalgae

Photobioreactor

Chlorella vulgaris

Cheese whey

Biomass

Çeşitli yetiştirme ortamlarında ek besin olarak kullanılan peynir altı suyunun biyodizel hammaddesi *Chlorella vulgaris*'in üretim potansiyeline etkisinin belirlenmesi

ÖZET

Mikroalgler, geleceğin yenilenebilir enerji kaynaklarından görülmesine rağmen, günümüzde büyük ölçekli üretimleri oldukça pahalı bir süreçtir. Mikroalgler tarımsal kaynaklı atık sularda yer alan kimyasalları ve ağır metalleri besin kaynağı olarak kullanarak üretimlerini devam ettirebilirler. Tarımsal atık sulardan önemli bir tanesi de peynir altı suyudur. Peynir atık suyu içerdiği yoğun protein nedeniyle mikroalg yetiştirilmesi için uygun bir besin kaynağı olarak kullanılabilir. Bu çalışmada, 500 ml'lik 4 farklı yetiştirme ortamı hazırlanarak 1)Mavi yeşil alg ortamı (BG11) *Chlorella vulgaris* 2) Mavi yeşil alg ortamı (BG11) Peynir altı suyu (CW) *Chlorella vulgaris* 3) Bold's Basal ortamı (BMM) *Chlorella vulgaris* and 4)Çeşme suyu (TW) *Chlorella vulgaris* ortamlarında üretilmeye çalışılmıştır. 21 gün süren denemeler sonucunda en yüksek, hücre sayısı, biyomas ve oransal yağ miktarı artışı Bold's Basal ortamı Peynir altı suyu ve *Chlorella vulgaris* ortamında sırasıyla 79.7x10⁶ cell mL⁻¹, 10.14 g L⁻¹ ve %20.7 olarak gerçekleşmiştir. Yapılan denemelerde peynir altı suyunun *Chlorella vulgaris* üretiminde besin kaynağı olarak kullanılabileceği sonucuna ulaşılmıştır. Sonuçlar istatistiksel analizlerle de desteklenmiştir.

Anahtar Sözcükler:

Mikroalg

Fotobiyoreaktör

Chlorella vulgaris

Peynir altı suyu

Biyomas

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1. Introduction

Microalgae is considered to be a feedstock for production of renewable energy. The lipid content of some microalgae can be as high as 60% (Oncel 2013; Mehrabadi et al., 2016). In recent years, there has been a great interest in microalgae due to this feature. Although microalgae are seen as fuel sources for the

future by many scientists, large-scale production of microalgae is still an expensive process (Slade and Bauen, 2013; Olkiewicz, Torres et al., 2016). For this reason, some methods are needed to reduce the costs of microalgae production (Acien et al., 2012; Kang et al. 2015). The most costly items of microalgae cultivation are food, artificial lighting and operational costs (Amaro et al., 2012; Panoutsou et al., 2013; Koc, 2015).

Microalgae can survive in very harsh conditions. Because the essential nutrients they usually need are heavy metals (Zhu et al., 2016). Heavy metals are abundant in industrial wastes, sewage waters and agricultural wastes (Wang et al., 2010; Cai et al., 2013; Hwang et al. 2016). These wastes can lead to considerable environmental pollution, if they are released to the environment without being filtered (Abdel-Raouf et al., 2012; Wu et al., 2014; Acien et al., 2016).

Nowadays with the industrial development, many agricultural wastes are emerging. Some of the main agricultural wastes are olive mill wastewater from olive oil factories, whey from milk processing plants, animal wastes from fattening stables and poultry farms (Zheng et al., 2011; Zeng et al., 2012; Lu et al., 2015). The common feature of these agricultural wastes is that they have plenty of nitrogen, phosphorus and organic carbon that microalgae can consume as nutrients (Aravinthan et al., 2014; Krustok et al., 2015; Lu et al., 2015).

For microalgae production, open pond and closed pond photobioreactors are used. Open pond from these photobioreactors is susceptible to the environmental effects and is not suitable for homogenous production. On the other hand, closed photobioreactors are more suitable for growing microalgae, however their operating costs are very high. An alternative for microalgae production in an enclosed environment is the use of polycarbonate bags. An important advantage of the use of polycarbonate bags is the ease with which microalgae can be taken in the growing medium. The illumination source can be placed around the polycarbonate so that the depth of light penetration can easily be adjusted. This allows biomass production even at very low light intensities (Raes et al., 2014; Gupta et al., 2015; Nwoba et al., 2016).

Olive mill wastewater, an important agricultural waste produced from olive oil factories, can be used as a fertilizer as it contains high amounts of phosphorus, nitrogen and organic carbon. It has been suggested by scientists that olive mill can be used as a nutrient source for microalgae cultivation as well. For this purpose, *Scenedesmus obliquus* was cultivated (Hodaifa et al., 2013). However the most frequently encountered issue with the use of agricultural wastes for microalgae cultivation is sterilization as fungi and bacteria have adverse effects on biomass production (Olivieri et al., 2012). Another agricultural waste product is whey which is generated during the production of cheese. Whey is a byproduct of cheese production that contains about 20% of milk proteins. Whey rich in chemical, physical and functional properties. Girard et al. (2014) conducted a study on growing microalgae using cheese whey. They reported that whey contributed positively to the increase in the number of *Scenedesmus obliquus* cells (Girard et al., 2014).

The objective of this study was to grow *Chlorella vulgaris* in a growing medium enriched with cheese whey. The purpose of this study is to collect data for

large-scale production according to the results obtained from the cultivation environments used in the experiments.

2. Material and Methods

2.1. Photobioreactor's design

Low cost polycarbonate bags were used to grow microalgae in a closed environment. An air pump was used to mix the microalgae in the growing medium and to supply the necessary air. The air pump was adjustable so that air can be supplied in polycarbonate tubes at desired amounts.

2.2. Lighting Source

Fluorescent lamps (39W/T5/GROLUX) were used to provide light. The illumination intensity of the selected fluorescent lamps was $170 \mu\text{W cm}^{-2} \text{ nm}^{-1}$. During the experiments, a light / dark cycle was applied to allow microalgae cells to regenerate themselves and produce lipid. For this purpose, the system was controlled by a programmable timer. A 16-h light 8-hour dark cycle was applied.

2.3. Penetration depth

Penetration depth of microalgae need to be adjusted carefully for photosynthesis (Lee and Palsson, 1995). For this purpose, fluorescent lamps were positioned around the photobioreactors so that light can reach the growing medium.

2.4. Growing medium

Chlorella vulgaris species microalgae was obtained from Ege Biotechnology Co (IZMIR, TURKEY). The algae were grown in BG11 culture growth medium consisted of KNO_3 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, H_3BO_3 , $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, EDTA. And Bold Basal Medium consisted of KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaNO_3 , K_2HPO_4 , NaCl , Na_2EDTA , $2\text{H}_2\text{O}$, KOH , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, H_2SO_4 (concentrated), Trace Metal Solution and H_3BO_3 . The most basic medium used in microalgae growing are blue green medium (BG11) and Bristol modified (BMM) growing medium (Vaiciulyte et al., 2014) In order to be able to investigate the effectiveness of whey in the study conducted, it has been mixed at certain rates in these growing environments.

Whey was obtained from the Dairy Factory of Ankara University for the experiments. Whey was kept at +4 °C until used in the experiments. Because of the nature of the wheywater, it was found that it contains large number of particles (sediments). These particles were separated by filtration (Tsolcha et al., 2016). The filtration process was repeated 3 times with a solid

separator. To remove the mixed aerosols from the flasks, autoclave with high vapor pressure was used and autoclave sterilization was provided for 3 hours. After autoclaving, the flasks that were kept in suitable sterile medium were then chilled. The contents of the whey used in the experiments are shown in Table 1.

The whey was sterilized for 3 hours in a high vapor pressure autoclave. After autoclaving, the flasks were allowed to cool in suitable sterile medium and were ready for microalgae (*Chlorella* sp) cultivation. The microalgae in the liquid state were transferred from the petri dish to the 1000 ml bottles to the BG11 liquid medium in a 1:10 dilution. After that, 4 different growing medium were prepared as shown in Table 2. Algae flasks were allowed to develop under sterile conditions in appropriate laboratory conditions, 220-240 V, 50-60 Hz fluorescent light.

Table 2. Growing medium.

1	BG11 + <i>Chlorella vulgaris</i> (250ml+250ml)
2	BG11 + Whey+ <i>Chlorella vulgaris</i> (250 ml+50 ml+ 200 ml)
3	BMM + Cheese Whey+ <i>Chlorella vulgaris</i> (250ml+50 ml+ 200ml)
4	TW + <i>Chlorella vulgaris</i> (250 ml+250 ml)

2.6. Determination of biomass concentration

A microscope and Thoma hemocytometer were used for microalgae cell counting (Ankara, TURKEY). In order to detect the cell count, 10 ml of material was mixed with 1 ml of isotonic diluents and cell counts were made under the microscope. The measurements were repeated every 3 days. For the cell count, the total number of cells in 1 ml was calculated by multiplying the number of cells in an area of 0.01 mm² on Thoma hemocytometer and 104 (Koc, 2015).

In order to determine the change in biomass, filter paper with a thickness of 8 µm was used. The filter papers were weighed before use, then 10 ml of sample was poured onto the filter paper. In order to determine the weight change, the paper was dried for 5 h at 75 °C in a vacuum oven. The change in biomass weight was calculated by subtracting the initial weight from the dried filter paper.

2.7. Statistical analysis

The statistical analysis of the data were made by using MINITAB. Statistical analysis was performed after the numbers were transformed to obtain a normal distribution in terms of cell intensity. For this purpose, statistical analysis was carried out after transformation of cell intensity values according to Log₁₀. The model used for the statistical analysis is shown in Eq. 1.

$$Y_{ijk} = \mu + a_i + b_j + e_{ijk} \quad (1)$$

Table 1. Composition of milk and whey(CW)

% by weight	Milk	Whey
Fat	4.7	0.05
Lactose(milk sugar)	4.5	5.0
Casein protein	2.7	0.10
Whey protein	0.55	0.65
Minerals	0.85	0.50
Minor components	0.20	0.30
Water	86.5	93.4

2.5. Growing medium

During the experiments, pH, temperature and EC values were measured on a daily basis.

Where, Y_{ijk} is the observation value, μ is the mean of the feature that is emphasized, a_i is the day effect ($i = 1, 2, \dots, 7$), b_j is the medium effect ($j = 1, 2, 3, 4$) and e_{ijk} is the error term. Subgroup comparisons were made according to Tukey ($P < 0.05$).

2.8. Determination of lipid

In the experiments, the micro algae were kept in the centrifuge at 3000 rpm for 5 minutes for 3 times. After centrifugation, sample material was kept at 60 °C for 12h and the amount of fat was determined using a Soxhlet device (Olivieri et al., 2011).

3. Result and Discussion

3.1. Result

Chlorella vulgaris and whey were mixed at certain ratios as shown in Table 3. Microalgae with an initial concentration of 25×10^6 cells mL⁻¹ was added to all medium and the highest concentration was obtained in sample 1 (50 ml CW + 200 ml BG11 + 10 ml *Chlorella vulgaris*) at 58×10^6 cells mL⁻¹. The view of the prepared preliminary experiments is shown in Figure 1.

cost polycarbonate bags were used to grow microalgae in a closed environment. An air pump was used to mix the microalgae in the growing medium and to supply the necessary air. The air pump was adjustable so that air can be supplied in polycarbonate tubes at desired amounts.

Table 3. Preliminary experiments.

s	Growing medium	Cell concentration (cell mL ⁻¹)
1.	50 ml CW + 200 ml BG11 + 10 ml <i>Chlorella vulgaris</i>	58x10 ⁶
2.	100 ml CW + 200 ml BG11 + 10 ml <i>Chlorella vulgaris</i>	49 x10 ⁶
3.	150 ml CW + 350 ml BG11 + 10 ml <i>Chlorella vulgaris</i>	42 x10 ⁶
4.	200 ml CW + 300 ml BG11 + 10 ml <i>Chlorella vulgaris</i>	31 x10 ⁶
5.	250 ml CW + 250 ml BG11 + 10 ml <i>Chlorella vulgaris</i>	28 x10 ⁶



Figure 1. Preliminary experiments.

Growing rate was more successful in the first and second growing medium than the other flasks. Maximum cell concentration was obtained at the end of the 3rd day in the flask containing 50 ml of whey and 200 ml of BG11. In the remaining 4 preliminary growing medium, there wasn't any increase. The most important factor for this reason that whey was very intense in the flasks (Giovanardi, Baldissertotto et al. 2016). The intensive whey prevented algae to photosynthesis by blocking the penetration of light. The algae culture could not grow, because molds were observed in the flasks mentioned above. In these environments molds cannot be prevented. Based on the results obtained from the preliminary experiments, 4 main experiments were conducted based on the highest cell concentration. During these experiments pH, EC and temperature were measured every 3 days. The measured values ranged from 8.85 to 9.29 for pH, 3200 to 2600 $\mu\text{S cm}^{-1}$ for EC and 29.5 to 32.2 °C for temperature.

3.2. Cell concentration

Cell count and weight measurements were performed to determine the cell concentration. At the end of the experiments, the highest cell number increase was obtained in the third growing medium (BMM + CW + *Chlorella vulgaris*) (79.7 x 10⁶ cell mL⁻¹). In other growing medium, only the number 4 decreased in the growing medium (TW + *Chlorella vulgaris*). The cell number also tended to increase in the first (BG11+*Chlorella vulgaris*) and second (BG11+CW+*Chlorella vulgaris*) growth medium (Figure 2).

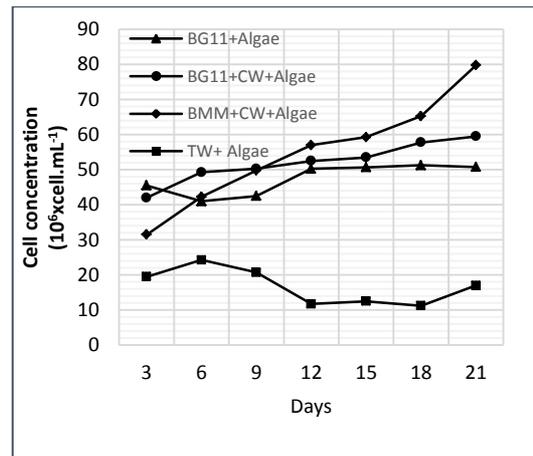


Figure 2. Cell concentration

For biomass production, the largest increase in the amount of biomass, was again obtained in third growing medium. During the experiments, the greatest biomass increase, was measured on the 18th day (12.60 g L⁻¹) (Figure 3).

During the experiments, the amount of lipid in the growing medium was determined as a percentage at the end of 21 day. As a result, the highest lipid content was obtained in BMM + CW + ALG medium with 20.7 %. BG11 + CW + Algae in 7.1 %, BG11 + Algae in 6.3 % and TW + Algae in 1.2 % (Figure 4).

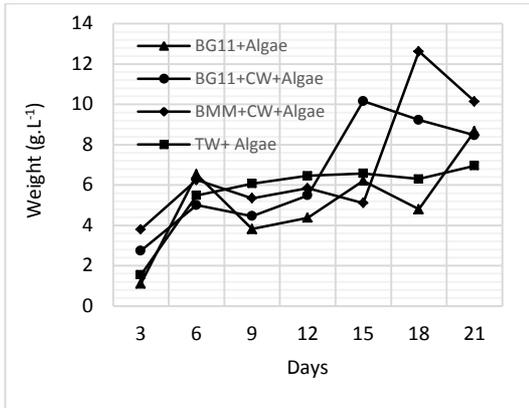


Figure 3. Evaluation of biomass weight

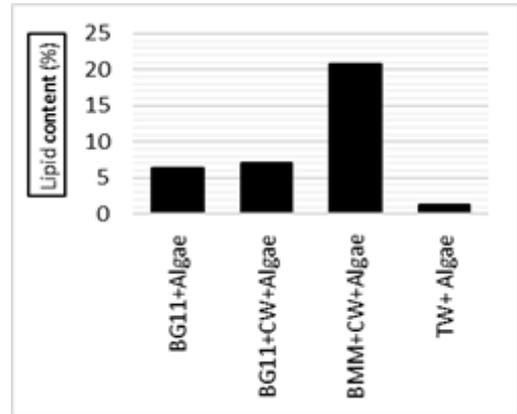


Figure 4. Lipid content (%)

Statistical significance of the treatments and interactions were summarized in Table 4. As a result of the statistical analysis, the effect of day factor on weight was significant ($P < 0.05$) and the effect of medium factor was negligible ($P > 0.05$). In comparison of subgroups; Day 1, Day 6 and Day 7 were different from $P < 0.01$. Day 1 was also found to differ from day 5 according to $P < 0.05$. The difference between the other

days is insignificant.

The effect of medium on cell intensity was found to be a significant at $P < 0.01$. The cell intensity density of TW + ALG is lower than other medium, and the difference was significant statistically. The differences among BG11 + ALG, BG11 + CW + ALG and BMM + CW + ALG medium were insignificant statistically ($P > 0.05$).

Table 4. Effects of treatment methods, whyeand interactions on the weight gain and cell count.

	n	Weight (gr)	Cell Concentration (Log10)	
Day		**	NS	
1	4	2.30±0.61 ^{Aa}	7.518±0.083	32,96 x10 ⁶
2	4	5.82±0.35 ^{Aa}	7.578±0.068	37,84 x10 ⁶
3	4	4.92±0.49 ^{Aa}	7.588±0.091	38,73 x10 ⁶
4	4	5.54±0.44 ^{Aa}	7.563±0.165	36,56 x10 ⁶
5	4	7.02±1.09 ^{Ab}	7.575±0.159	37,58 x10 ⁶
6	4	8.24±1.73 ^{Bb}	7.583±0.179	38,28 x10 ⁶
7	4	8.56±0.65 ^{Bb}	7.653±0.146	44,98 x10 ⁶
Growth medium		NS	**	
BG11+Algae	7	5.07±0.90	7.6743±0.0156 ^{Aa}	47,24 x10 ⁶
BG11+CW+Algae	7	6.51±1.05	7.7129±0.0190 ^{Aa}	51,63 x10 ⁶
BMM+CW+Algae	7	7.02±1.20	7.7243±0.0491 ^{Aa}	53,00 x10 ⁶
TW+Algae	7	5.63±0.70	7.2057±0.0500 ^{Bb}	16,06 x10 ⁶

3.3. Discussion

Microalgae were cultivated in an indoor environment made of polycarbon. The risk of contamination of the growing medium with environmental factors were reduced (Singh and Dhar, 2011). Polycarbonate bags offer low cost systems for microalgae production. Polycarbonate bags can provide suitable lightning source for microalgae optimally. Optimum penetration depth can be adjusted by using large surface area (Lee et al., 2014). Polycarbonate bags are very convenient for harvesting microalgae. Production in polycarbonate bags reduce water consumption because microalgae are grown in a closed

environment (Tu et al., 2016). One of the most important and expensive parameters in microalgae cultivation is lighting (Richmond, 2004). The wavelength of illumination sources may cause characteristic changes in the microalgae structure (Glemser et al., 2016) The wavelength of the red and blue LED lamps have different effects on the microalgae cell counts and weights. Blue LED lamps are effective increase in weight of microalgae cells. Red LED lamps are effective in increasing the number of cells (Koc et al., 2013). In the experiments, fluorescent lamps (170 $\mu\text{W cm}^{-2} \text{nm}^{-1}$) were used as an illumination source. Fluorescent lamps consume more energy than LED lamps. However, fluorescent lamps have been

used effectively in this study because they are closer to daylight and contain wavelengths required for photosynthesis (Schulze et al., 2016). Fluorescent lamps can provide effective penetration depth. The lamps were located at a distance of about 10 cm near the photobioreactors for effective illumination. Lighting sources are not located too close to the microalgae due to temperature effects (Lee and Palsson 1995).

In order to determine the effect of whey on the biodiesel potential of *Chlorella vulgaris*, CO₂ is not supplied to the system but only air is mixed. According to the literature, the lipid content of *Chlorella vulgaris* is 20-53% (Yeh and Chang 2012). For this species used in experiments, the highest lipid percentage was found as 20.7%. Higher lipid ratios can be achieved by adding CO₂ to the BMM medium with cheese whey in the growing media (Adamczyk 2016, Ismail 2016, Parupudi 2016).

4. Conclusion

In this study, the possibility of using whey as an additional nutrient in the biomass production of *Chlorella vulgaris* was investigated. Positive results were achieved after 21 days out of four different growing medium created for this purpose. The highest increase in cell count, biomass weight and lipid ratio in whey + BMM growing medium was 79.7x10⁶ cells mL⁻¹, 12.60 g L⁻¹ and 20.7%, respectively. As a result of this study, whey can be used together with BMM as an additional nutrient to cultivate *Chlorella vulgaris*. Higher amount of lipid rates can be obtained for biomass with CO₂ addition to the growth medium.

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