

Increasing the Protein Amount of *Chlorella vulgaris* [Chlorophyta] Strains Isolated from Different Fresh Water Ponds

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Abstract: *Chlorella vulgaris* is a single-cell, spherical green algae and one of the microalgae on which many applied studies are conducted. In the present study, five strains displaying fast and efficient reproduction were chosen among 11 *C. vulgaris* strains isolated from different fresh water ponds and their cell numbers and the amount of chlorophyll a, protein, lipid, cellulose and carbohydrate were examined. The main goal of the study is to investigate increasing the biochemical contents especially the protein content of *C. vulgaris* strains in different mediums. In the present study, cell densities were determined through cell count for five days. In parallel with cell count, their chlorophyll a content was determined. The highest cell density was observed with *C. vulgaris* TOH (Tourism and Hotel Management Pond) strain as 5.5×10^4 h/mL, and the chlorophyll a content as 4.3×10^2 mg/m³. The highest intracellular protein amount was determined with *C. vulgaris* GUH (Gazi University Rectorship Pond) (0.061 g/100 mL) and the highest lipid amount was attained with *C. vulgaris* UIK (Ulus Construction Well) strain as 0.019 g/100 mL. The process of increasing the intracellular protein amount in *C. vulgaris* GUH strain was carried out in Prat, Yagojinski and Chlorella medium. The results indicated that Chlorella medium increased the intracellular protein amount.

Key words: *Chlorella vulgaris*, chlorophyll a, microalgae, biomass, biochemical analysis.

1. Introduction

The microalgae are the organisms which synthesize organic substance in aqueous media, constitute the first circle in the food chain and produce atmospheric oxygen [1, 2]. They are used commercially in fields such as food and feed industry due to the fact that they own metabolites such as protein, lipid, carbohydrate and carotenoid [3, 4]. Besides, the microalgae contain A, C, B₁, B₂, B₆, niacin, iodine, potassium, iron, magnesium and calcium abundantly [5]. These photosynthetic microorganisms constitute the living materials on which scientists study mostly upon subjects such as waste water treatment [6], bioremediation applications [7] and biofuel production [8, 9].

C. vulgaris is a single-cell and spherical green algae utilized in order to find out the potential use of microalgae in applied research [10]. *C. vulgaris* are

effective microorganisms for bio-fixation of carbon concentrations due to their high photosynthetic potential resulting from chlorophyll a and b content in them [11]. Meanwhile, the cultures of *C. vulgaris* are made with respect to reproduction of healthy secondary metabolites as they are robust and fast-growing species of the microalgae. *C. vulgaris* has potential in food and other industries such as biofuel, cosmetics and pharmaceutical industries [12]. *C. vulgaris* biomass when dried consists of approximately 40% protein, 25% oil, 20% carbohydrate, 5% fiber and 10% minerals and vitamins despite its highly efficient photosynthesis which allows it to produce more oil and protein than most other plants [13, 14]. Besides, *C. vulgaris* is commercially of great significance due to the fact that it contains various enzymes, carotenes and chlorophyll at high levels. Chlorophyll is a pigment which is responsible for converting luminous energy into chemical energy through photosynthesis

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[15]. Mulders, K. J., et al. [16] identified the structure of chlorophyll as “a hydrocarbon tail connected to chlorin, an aromatic ring that contains a tetrapyrrole in which the central magnesium ion is bound to four pyrrole rings”. Chlorophyll is photoreceptor of photosynthesis and has antioxidant and antimutagen characteristics. It has various industrial areas of use such as an additive in pharmaceutical and cosmetic products and as natural food colorant [17, 18].

C. vulgaris strains from different ordinary ponds in Ankara, Turkey were isolated in previous studies. They were determined the best culture media and reproduction conditions in the laboratory. In the present study, five strains displaying the fastest and the most efficient reproduction were chosen and their capacities to produce chlorophyll a, protein, lipid, cellulose, carbohydrate and ash were examined. The main goal of the study is to investigate increasing the biochemical contents especially the protein contents of *C. vulgaris* strains under optimum culture conditions and in different mediums.

2. Material and Methods

2.1 Test Microorganisms

The samples collected from different ponds were brought to incubation at room temperature after inoculation at pre-enrichment nutrition media. *C. vulgaris* was identified at species base with microscopic examination after incubation [19, 20]. Cultures were isolated by purification from one colony as inoculation to solid media. The colonies breaded in Petri dishes were taken to liquid media and the purification of cultures was completed. Those cultures were screened considering their breeding characteristics in laboratory conditions in a previous study and five of them were chosen to be used in further studies with the best and the most rapid production rates. The coding of *C. vulgaris* strains were listed as follows: *C. vulgaris* GUH (Gazi University Rectorship Pond), *C. vulgaris* AUH (Ankara University Pond), *C. vulgaris* TOH (Tourism and Hotel Management Pond), *C. vulgaris*

GUMSH (Gazi University Medico Social Pond) and *C. vulgaris* UIK (Ulus Construction Well).

2.2 Culture Conditions

Semi-continuous culture system was utilized in reproduction of the cultures. The cultures were made in *Chlorella* nutrient media in such a way that would be suspended culture with 270 mL medium + 30 mL and they were placed into a shaker. The pH of nutrient media was adjusted as 6.5-7. The light source ($150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was horizontally placed to a distance of 22 cm away from the cultures. The implementation of 16:8 light/dark photoperiod was applied on cultures and they were cultivated under at 22-25 °C room temperature. The chemical compositions belonging to the nutrient media used during the study are displayed in Table 1.

2.3 Determination of Cell Density

The cell densities were determined through counting cells falling to 16 squares on Thoma slide. Cell countings were made at the beginning of cultivation process within hours of 0, 24, 48, 96 and 120. The cell densities were calculated through Eq. (1) [21].

$$\text{CN} = \frac{\text{T} \times 4000}{16} \quad (1)$$

(CN: Cell number; T: Total cell number falling on 16 squares; 4000: Volume of one square).

2.4 Determination of Dry Weight

The 50 mL samples were filtered via Whatman GF/C filter papers and dried at 60 °C in incubator and they were weighed [22]. Dry weight determination was made on seventh day following the cultivation.

2.5 Determination of Chlorophyll a

By using Whatman GF/C filter paper, 150 mL's of cultures were filtered. The filter papers including *C. vulgaris* cultures were dried at room temperature during 3-4 hours. Then, they were cut into pieces and

Table 1 Chemical composition of the culture broths tested.

Macroelements	Culture broth compositions		
	Chlorella (g/L)	Prat (g/L)	Yagojinski (g/L)
MgSO ₄ ·7H ₂ O	5.0	0.01	-
K ₂ HPO ₄	-	0.01	-
KH ₂ PO ₄	2.5	-	-
NaCl	2.0	-	-
CaCl ₂	0.022	-	-
KNO ₃	-	0.1	0.5
MgSO ₄	-	-	0.1
Na ₂ HPO ₄	-	-	0.2
FeSO ₄	-	-	0.002
FeCl·6H ₂ O	-	0.001	-
Sodium iron EDTA	0.004	-	-
Urea	0.4	-	-

put into 90% acetone solutions. 0.3 g of MgCO₃ was added into these solutions in order to prevent degradation of chlorophyll and was brought to extraction at 3-4 °C under dark conditions for 24 hours. After the extraction, the extracts were filtered with filter paper and the absorbance values were measured through spectrofotometer. The chlorophyll a values were determined as described by Chia, M. A., et al. [23]. During five days, chlorophyll a amounts were determined together with cell counts. Both cell densities and chlorophyll a measurements were carried out in three parallels and the results were given by taking the average.

2.6 Biochemical Analyses

Mass cultures of five cultures were made in Chlorella nutrient media. The protein, lipid, cellulose, carbohydrate and ash analyses of cultures were made in Tubitak Marmara Research Center Food Science and Technology Institute in accordance with AOAC (Association of Official Analytical Chemists) International Standard methods. The values were given out of 100 mL liquid culture. The carbohydrate values involve mono, di and polysaccharide values.

3. Results and Discussion

The present study was conducted as based upon the

intracellular biochemical change observed during cultivation of *C. vulgaris* strains isolated from the samples collected from fresh water ponds. During the study, the cell densities were determined through cell count and chlorophyll a determination was made in parallel with cell count. The highest cell density was found in *C. vulgaris* TOH strain as 5.5×10^4 h/mL with chlorophyll a 4.3×10^2 mg/m³, the dry weight of *C. vulgaris* GUMSH strain was determined as 0.03 g/50 mL (Tables 2 and 3).

The biochemical analyses of mass cultures belonging to 5 strains produced in Chlorella nutrient media were conducted and the results are presented in Table 4. *C. vulgaris* GUH (0.061 g/100 mL) strain was observed to have the highest protein amount which is an important intracellular metabolite. Being another valuable metabolite, the highest lipid amount was found in *C. vulgaris* UIK with (0.019 g/100 mL).

Furthermore, the study focused upon whether intracellular protein amount in *C. vulgaris* GUH strain could be increased through use of different nutrient media. Therefore, mass culture of *C. vulgaris* GUH strain was made in Prat, Yagojinski and Chlorella media. The analysis results are displayed in Table 5. The analysis results indicated that Chlorella medium increased intracellular protein amount.

Table 2 Cell number and dry weight of *C. vulgaris* cultures.

Strains	Total number of cells (cell/mL)					Dry weight g/50 mL
	0 h	24 h	48 h	96 h	120 h	
TOH	$3.2 \times 10^4 \pm 0.21$	$3.7 \times 10^4 \pm 0.35$	$4.4 \times 10^4 \pm 0.5$	$5 \times 10^4 \pm 0.28$	$5.5 \times 10^4 \pm 0.21$	0.029 ± 0.0006
AUH	$3.3 \times 10^4 \pm 0.3$	$4 \times 10^4 \pm 0.35$	$4.7 \times 10^4 \pm 0.3$	$5 \times 10^4 \pm 0.2$	$5.4 \times 10^4 \pm 0.05$	0.026 ± 0.005
GUH	$3.2 \times 10^4 \pm 0.25$	$4.1 \times 10^4 \pm 0.26$	$4.6 \times 10^4 \pm 0.62$	$5 \times 10^4 \pm 0.52$	$5.1 \times 10^4 \pm 0.47$	0.024 ± 0.004
UIK	$1.9 \times 10^4 \pm 0.2$	$2.9 \times 10^4 \pm 0.65$	$4.3 \times 10^4 \pm 0.7$	$4.6 \times 10^4 \pm 0.15$	$5 \times 10^4 \pm 0.37$	0.022 ± 0.0006
GUMSH	$3.3 \times 10^4 \pm 0.21$	$3.8 \times 10^4 \pm 0.4$	$4.3 \times 10^4 \pm 0.21$	$4.5 \times 10^4 \pm 0.43$	$4.8 \times 10^4 \pm 0.32$	0.03 ± 0.006

(\pm Standard error, n = 3).

Table 3 Chlorophyll a determination of *C. vulgaris* cultures.

Strains	Chlorophyll a content of strains (mg/m ³)					Vol. weight (g/50 mL)
	0 h	24 h	48 h	96 h	120 h	
TOH	$1.3 \times 10^2 \pm 0.14$	$1.2 \times 10^2 \pm 0.23$	$3.4 \times 10^2 \pm 0.14$	$3.9 \times 10^2 \pm 0.14$	$4.3 \times 10^2 \pm 0.07$	0.05 ± 0.013
AUH	$0.9 \times 10^2 \pm 0.02$	$1.4 \times 10^2 \pm 0.15$	$1.6 \times 10^2 \pm 0.21$	$2.7 \times 10^2 \pm 0.21$	$3.5 \times 10^2 \pm 0.35$	0.047 ± 0.004
GUH	$0.5 \times 10^2 \pm 0.14$	$1.4 \times 10^2 \pm 0.07$	$1.5 \times 10^2 \pm 0.21$	$2.3 \times 10^2 \pm 0.07$	$3.4 \times 10^2 \pm 0.42$	0.032 ± 0.002
UIK	$1.1 \times 10^2 \pm 0.21$	$1.5 \times 10^2 \pm 0.07$	$2.1 \times 10^2 \pm 0.14$	$2.4 \times 10^2 \pm 0.35$	$3.2 \times 10^2 \pm 0.07$	0.044 ± 0.02
GUMSH	$1.5 \times 10^2 \pm 0.07$	$1.5 \times 10^2 \pm 0.35$	$2.3 \times 10^2 \pm 0.14$	$2.7 \times 10^2 \pm 0.21$	$2.9 \times 10^2 \pm 0.14$	0.045 ± 0.02

(\pm Standard error, n = 3).

Table 4 Biochemical analysis results of *C. vulgaris* cultures.

Strains	Protein g/100 mL	Lipid g/100 mL	Cellulose g/100 mL	Carbohydrate g/100 mL	Ash g/100 mL
GUH	0.061	0.010	0.040	0.178	0.041
GUMSH	0.048	0.012	0.026	0.051	0.033
TOH	0.045	0.010	0.045	0.702	0.038
AUH	0.044	0.010	0.032	0.790	0.034
UIK	0.037	0.019	0.025	0.627	0.019

Table 5 Biochemical analysis results of GUH culture produced in three different nutrient media.

Culture media	Protein (g/100 mL)	Lipid (g/100 mL)	Cellulose (g/100 mL)
Chlorella medium	0.4453	0.0360	1.132
Yagojinski medium	0.4065	0.0177	1.197
Prat medium	0.0969	0.1128	1.450

Phytoplankton need to take macronutrient elements (i.e. carbon, nitrogen, phosphorus), basic ions (i.e. Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, SO₄²⁻) and micronutrient metals (i.e. iron, manganese, zinc, cobalt, copper, molybdenum, nickel and cadmium) in order to maintain their growth and division in aquatic environments [24, 25]. The media combination has great significant influence both upon growth rate and final concentration of microalgae while cultivating the microalgae in culture medium and the amount of those elements varies by the nutrient media [26]. Some essential elements such as N, P, K, Mg, Ca, S, Fe, Cu,

Mn and Zn are required in growth of the microalgae in culture medium and those elements are utilized in the form of salts [27].

The biochemical compositions of algae (such as protein, carbohydrate, lipid, amino acid and pigments composition) change with respect to temperature, light, nutrient media components, strain characteristics and growth phase. The microalgae can grow under different temperatures and the temperature affects the structure of cell compounds, the chemical reactions, enzymatic activity, membrane liquidity, the efficiency of electron transfer chain and other metabolic

mechanisms [28]. The temperature in culture media in the present study was determined to be between 22-25 °C and *C. vulgaris* cultures showed good development under that temperature. The light is the energy source of photosynthesis and a necessary factor for transforming inorganic carbon into organic molecules and for growth and obtaining energy. During cultivation of the cultures, 12:12 or 18:6 h light/dark cycles were generally reported to be more efficient when compared to the practices with 24:0 and 6:18 h [29]. Therefore, the cultures were applied 18:6 h light/dark cycles.

Nitrogen is significant substance for synthesis of protein, nucleic acids and chlorophyll molecules [30]. It was observed that the lack of nitrogen in the medium caused a decrease in chlorophyll content of cultures [31]. Urea ($\text{CH}_4\text{N}_2\text{O}$) and EDTA (Ethylenediaminetetraacetic Acid) is the nitrogen source of *Chlorella* medium and they are responsible for protein synthesis. Phosphorus is another significant nutrient and necessary for many phosphorylation-dependent events such as ATP (Adenosine Triphosphate) synthesis and the Calvin cycle. Therefore, the lack of phosphorus affects not only the synthesis of chlorophyll but also the growth and metabolism of cells [32, 33]. The KH_2PO_4 in *Chlorella* nutrient medium and K_2HPO_4 in Prat nutrient medium is the source of phosphate for algal growth. Fan, J., et al. [34] conducted a study upon *Chlorella pyrenoidosa* and reported that the low phosphorus concentration for seven days decreased biomass.

The magnesium ions are central atom of chlorophyll. They play a significant role in growth of microalgae as a cofactor of some key enzymes in the metabolic pathway [35]. The researchers put forward that the intracellular chlorophyll content decreased in the absence or lack of magnesium, which resulted in a negative impact upon photosynthesis amount in *Chlorella sp.* [15, 36]. Magnesium source of growth media used in authors' study was MgSO_4 . Iron is one

of the vital elements in algal growth whose deficiency results in growth retardation [37]. FeSO_4 , $\text{FeCl}\cdot 6\text{H}_2\text{O}$ and sodium iron EDTA in the composition of the nutrients served as an iron source for culture in this study.

The cell densities and chlorophyll a amounts of *C. vulgaris* strains were presented respectively: the highest value of cell density in TOH strain with 5.5×10^4 h/mL, chlorophyll a 4.3×10^2 mg/m³, following that, the cell density in AUH strain as 5.4×10^4 h/mL and chlorophyll a as 3.5×10^2 mg/m³; the cell density in GUH strain as 5.1×10^4 h/mL, chlorophyll a as 3.4×10^2 mg/m³; the cell density in UIK strain as 5×10^4 h/mL and chlorophyll a as 3.2×10^2 mg/m³ and lastly the cell density in GUMSH strain as 4.8×10^4 h/mL and chlorophyll a as 2.9×10^2 mg/m³ (Tables 2 and 3). On the other hand, the dry weight values were determined as 0.03 g/50 mL in GUMSH strain which is followed as TOH 0.029 g/50 mL, AUH 0.026 g/50 mL, GUH 0.024 g/50 mL and UIK 0.022 g/50 mL (Table 2). Sharma, R., et al. [38] reported cell density of *C. vulgaris* as 4.4×10^6 cells/mL, dry weight as 30.2 mg/50 mL and chlorophyll a amount as 2.16% under different temperatures and light regimes. Sostarie, M., et al. [39] carried out a study on production of *C. vulgaris* with different carbon resources by using Jaworski medium and reported that the cell number rose to maximum (7.8×10^6 cell/mL) at the end of seventh day. Both the results obtained by Sharma, R., et al. [38] and by Sostarie, M., et al. [39] showed parallelism with the results obtained in the present study with regards to cell number and chlorophyll a. Although the dry weight results obtained by Sharma, R., et al. [38] are found higher, it is supposed that this result stemmed from the characteristics of nutrient media and strains used.

In *Chlorella* nutrient medium, the highest protein amount of strains was observed with *C. vulgaris* GUH (0.061 g/100 mL) and the lowest was with *C. vulgaris* UIK (0.037 g/100 mL) and the other strains were realized to be between those two values. The

carbohydrate amounts were the highest in *C. vulgaris* AUH (0.79 g/100 mL) strain and the lowest in *C. vulgaris* GUMSH strain (0.051 g/100 mL) while the highest lipid amount was observed in *C. vulgaris* UIK strain (0.019 g/100 mL) and the lowest in *C. vulgaris* GUH, TOH, AUH strains (0.010 g/100 mL). Besides, the biochemical composition of *C. vulgaris* was found as protein ($39.2 \pm 1.1\%$), carbohydrates ($24.5 \pm 0.3\%$), lipids ($25.3 \pm 0.1\%$) in BG-11 nutrient media and as protein ($37.2 \pm 0.8\%$), carbohydrates ($27.7 \pm 0.2\%$), lipids ($26.8 \pm 0.2\%$) in Kolkwitz nutrient media [22, 40]. The growth, biomass production and biochemical composition of *C. vulgaris* cultures were examined in three different media (LC Oligo, Chu 10 and WC media) and the highest cell density (2.74×10^6 cells/mL) and chlorophyll a ($1 \mu\text{g/mL}$) were observed in LC Oligo nutrient medium. The protein production was found 50% ($7.0 \text{ mg}\cdot\text{L}^{-1}$) in Chu medium. The highest carbohydrate production ($7.36 \mu\text{g}\cdot\text{mL}^{-1}$) was observed with the cultures cultivated in LC Oligo under the same conditions. The percentage of total lipid yield was stated to be 10.7 g per 100 gr dry algae [22]. The results obtained by those researchers showed similarities with the results obtained in the present study.

The differences in biochemical compositions stemming from growth phase are generally based upon the age of culture and nutrient problem especially in the case of semi-continuous production. The algal cultures experience nutrient problem in stationary phase of their growth. In this situation, while the total amount of lipid and carbon dioxide increases, the protein amount decreases [41]. The present study aiming to increase intracellular protein amount was conducted while the microalgae were in growth phase because of the reason mentioned above.

In the other part of the study oriented towards increasing intracellular protein amount through use of different nutrient media, the mass culture of *C. vulgaris* GUH strain with the highest protein amount was made in Prat, Yagojinski and Chlorella media.

Chlorella medium was observed to increase intracellular protein amount (Table 5).

4. Conclusion

C. vulgaris is a microalgae species on which many scientific studies are conducted due to fast reproduction cycle and easy cultivation under laboratory conditions. Besides, they are used in food and drug industries because of their nutritional value, natural pigments and antioxidant activity. Taking all those characteristics into consideration, the aim of the present study is to determine the appropriate strain by optimizing the best culture condition for obtaining high biomass, chlorophyll content, total protein, carbohydrate and lipid. Sub-culture reproduction of 11 strains isolated from fresh water ponds and cultivated in semi-continuous culture systems, the morphological sizes and numbers of their cells, and their biomass were investigated. Five strains having high reproduction and photosynthesis rate, resistant to light and contamination were chosen and their biochemical structures were examined under different nutrient media. Consequently, it can be stated that Chlorella nutrient medium increases the intracellular protein amount under optimum culture conditions.

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