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RESEARCH ARTICLE

Investigation of autotrophic, heterotrophic, and mixotrophic modes of cultivation on lipid and biomass production in *Chlorella vulgaris*

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ABSTRACT

Background: The cultivation mode plays a great role in biomass production and composition in the green microalga *Chlorella vulgaris*. **Aims and Objectives:** The effects of three different cultivation modes including autotrophic, heterotrophic, and mixotrophic growth conditions on the biomass and lipid production and also on the biomass composition in the unicellular photosynthetic microalga *C. vulgaris* were investigated. **Materials and Methods:** BG-11 culture medium was used for preservation and analysis of the studied microalgal strain. Effects of three different cultivation strategies including autotrophic, heterotrophic, and mixotrophic modes of cultivation were investigated on biomass and lipid production in *C. vulgaris*. **Results:** The maximum amount of cell dry weight was obtained in mixotrophic mode (3.91 g L^{-1}) while autotrophic and heterotrophic modes achieved a final cell dry weight of 2.47 g L^{-1} and 1.52 g L^{-1}, respectively. Besides, the biomass production in mixotrophic was increased 158% and 257% in 21 days, compared with autotrophic and heterotrophic conditions. The heterotrophic cultivation mode achieved the highest lipid contents (48.68%) which was 110.01% and 143.13% times higher than the mixotrophic (44.25%) and autotrophic (34.01%) conditions. The protein accumulation in autotrophic mode with 41.7% had the highest value. **Conclusion:** Considering the biomass produced in mixotrophic condition in the studied microalgal strain, this cultivation mode is recommended for lipid production which can be used for green energy production.

KEY WORDS: Biofuel; Biomass; *Chlorella vulgaris*; Cultivation Mode; Lipid

INTRODUCTION

Chlorella vulgaris is fast-growing green microalga which produces high levels of biomass and lipids under natural conditions.[1] *C. vulgaris* contains 50–60% proteins and 19 amino acids that among them, there are eight essential amino acids, Vitamins (B_6, B_{12}, E, and C), minerals such as potassium, sodium, magnesium, iron, copper, calcium, and selenium, methylcobalamin, sporopollenin, and unsaturated fatty acids (α- and γ-linoleic acid). This single-celled microalga is widely considered as a food source due to the rapid growth and valuable biomass content composed of the all essential ingredients for the proper functioning of the human body.

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Many researchers have reported the medicinal effects of *Chlorella* species including reducing the blood glucose in diabetic mice and lowering the blood pressure and lipids, anticancer effects, and also cerebral strokes.[2-4] *C. vulgaris* extract can stimulate the synthesis of collagen in the skin and

reduce wrinkles.[5] Furthermore, it could play a great role in the body detoxification.

The lipids from microalgae could be used in production of biofuel, especially biodiesel.[6] The microalgal protein could be used in food and pharmaceutical industries to production food supplements, animal feed and aquaculture, and also pigment production.

In addition to the essential chemical factors including macro- and microelements, physical factors such as light, temperature, aeration, and pH should also be considered for optimal growth of microalgae.[7] Therefore, the microalgal culture conditions should be optimized to produce higher levels of biomass, lipids, and proteins.

In autotrophic mode of cultivation, the microalgal strain uses CO_2 and the solar energy to generate the biomass and valuable products.[8] Although the light penetration and the cell concentration have a reverse relationship. This phenomenon causes the low-density biomass of microalgae in autotrophic culture.[9] Heterotrophic condition dispels the requirement for light and increased cell density,[10] but the cost of the organic carbon sources (usually in the form of glucose or acetate) is high. In mixotrophic culture, the light is the main source of energy; however, an organic carbon source is added to the culture medium simultaneously.

In the present study, we investigate the effect of autotrophic, heterotrophic, and mixotrophic growth conditions on *C. vulgaris* growth, biomass production and productivity, and also biomass composition including the total lipid and protein analyses during three different types of cultivation. Besides, some bioinformatics studies were performed to confirm the identification of the naturally isolated microalgal strain.

MATERIALS AND METHODS

Isolation and Purification of Microalga

C. vulgaris MCCS AB127 strain was isolated from soil samples collected from the rice fields in Marvdasht city, Fars province, located at the south of Iran, and cultured on BG-11 culture medium.[11] After 10 days of incubation, a single colony was isolated and inoculated in 50 mL conical flask containing BG-11 medium and incubated in an orbital incubator shaker (PECO, Iran), at 25°C, under the light intensity of 60 mol m^{-2} s^{-1} with 130 rpm agitation.

Growth Measurement

The microalgal growth trend was observed for 21 days. The experiment was performed in 500 mL Erlenmeyer flasks. The microalgal biomass was measured using dried cell weight method. For this study, 5 mL of each culture was centrifuged for 5 min at 6000 g. The obtained cell pellets were washed with an isotonic saline solution and centrifuged again for removing the possible impurities. After it, the *C. vulgaris* pellets were dried overnight at 88°C. Besides, the direct cell counting method using Neubauer hemocytometer was also employed for cell growth measurement. Each study was designed and performed in triplicates, and the average values were reported. The sampling procedure was performed every day for cell counting and every 3 days for dried cell weight method for each experiment during 21 days of study.

Ribosomal Gene Extraction, Polymerase Chain Reaction (PCR), and Sequencing

C. vulgaris identification was performed using morphological and 18S rRNA gene sequencing studies. The genomic content of the isolated microalgal strain was extracted using the heat shock method (99°C for 2 min). The 18S rRNA encoding gene was amplified using the universal primers against 18S rRNA encoding genes[12] using PCR method, according to a previously described protocol.[13] Moreover, the morphologic methods[14] were also exploited to confirm the identification and sequencing studies.

Bioinformatics Studies

The identified 18S rRNA gene sequence was aligned and compared with seven related *C. vulgaris* strains from GenBank database of the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MAFFT (version 7) multiple sequence alignment software was exploited to create the multiple sequence alignments.[15] The CLC sequence viewer software (Qiagen, Aarhus, Denmark) version 7.8.1 was employed to investigate and shading the conserved domains.[16] MEGA software version 7[17] was used for phylogenetic studies based on the obtained 18S rRNA gene sequencing data.

Analytical Methods

The total lipid contents were extracted and measured from the finally obtained biomass in each experiment using a previously reported method.[18] The lyophilized biomass of *C. vulgaris* strain was used for this analysis. Pure olive oil was used to plot the standard curve for lipid determination. The total protein content of the microalgal strain was investigated using the Bradford method in 96-well plates. Briefly, 20 mg of the dried microalgal biomass was solved in 1 mL NaOH 1N and incubated at 100°C in hotplate. The resulting solution was centrifuged, and then, 0.1 mL of the supernatant solution was mixed with 5 mL Bradford reagent. The absorbance was measured at 595 nm after 2 min. The total carbohydrate content was quantified using the phenol-sulfuric acid method.[19] The glucose solution with different concentrations was used to plot the standard curve.

Statistical Analysis

The GraphPad Prism version 7.00 (GraphPad Software, La Jolla, California, USA) was employed for statistical analysis. The significant differences (n = 3) were analyzed using ANOVA with the statistical difference level of 5%.

RESULTS

Identification of the Microalgal Strain

Multiple sequence alignment studies showed a high similarity (100%) between the amplified 18S rRNA gene in the studied strain (679 bp) with some previously reported 18S rRNA sequences of other *C. vulgaris* strains in NCBI database. Moreover, the conserved domains among the investigated strains were identified in a color scale [Figure 1]. The blue residues present the least conserved domains while the white residues depict the most conserved domains. Besides, the name of each sequence is presented at the left side of each row. Furthermore, the numbers above the alignment columns represent the position of each residue. Under the eight investigated sequences, the consensus sequence is indicated. Moreover, a yellow-colored bar in percent scale indicates the conservation level for each studied domain.

Figure 2 is showing the molecular phylogenetic analysis of eight studied *C. vulgaris* strains. The evolutionary history was inferred using the maximum likelihood method. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The analysis involved eight nucleotide sequences (accession numbers in parenthesis). All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted in MEGA 7 software. The phylogenetic analysis revealed that there is a close genetic relationship between the isolated *C. vulgaris* strain in this study and *C. vulgaris* isolate L5. The identified *C. vulgaris* strain was deposited in microalgal culture collection of Shiraz University of Medical Sciences (MCCS) as *C. vulgaris* MCCS AB127 for preservation, cultivation, and future studies.

Growth Measurement Studies

The cell growth trend was monitored every 3 days using dried cell weight and also every day for direct cell counting methods

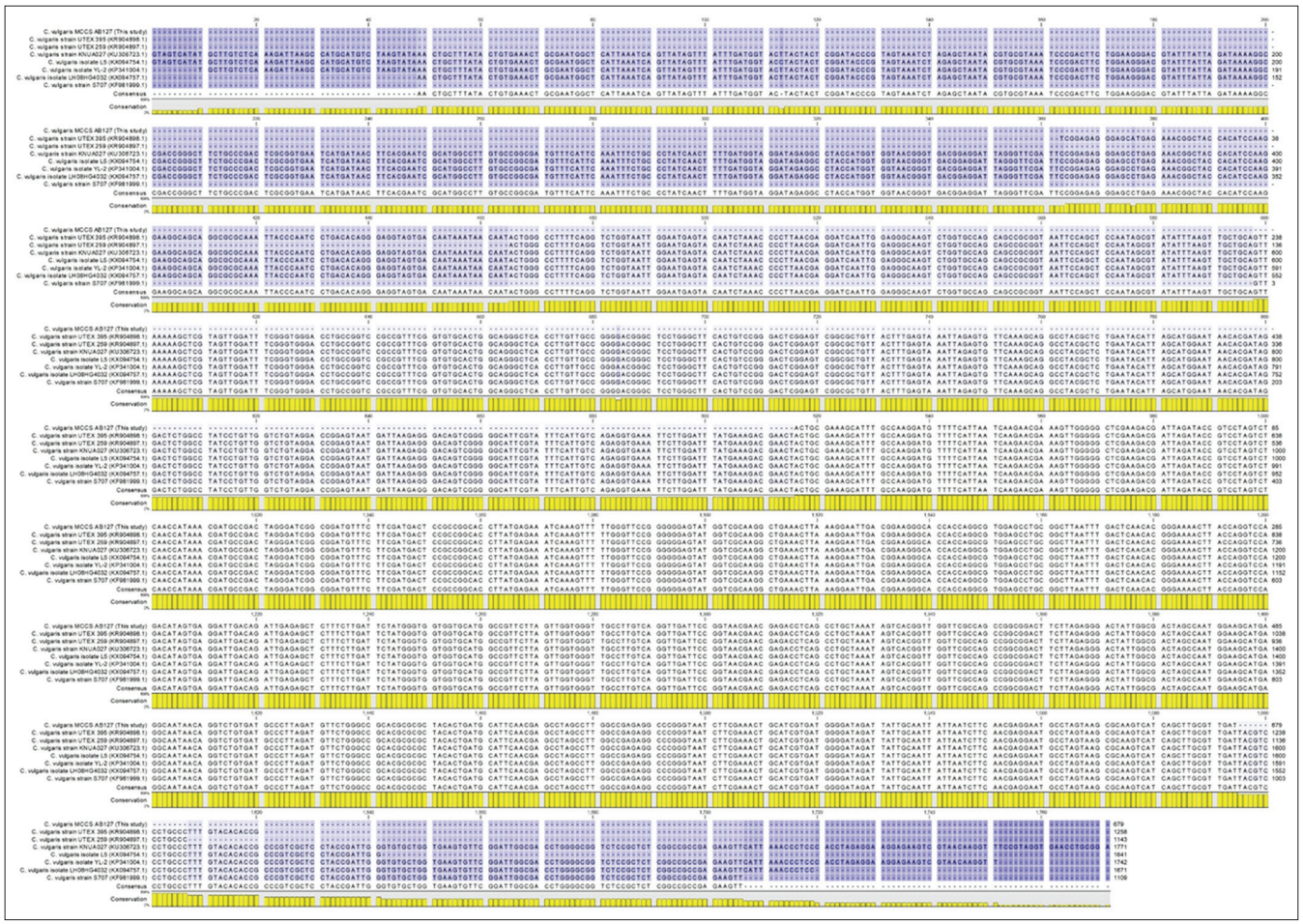

Figure 1: The tabular format of a multiple alignment from *Chlorella vulgaris* MCCS AB127 strain and seven related *C. vulgaris* strains retrieved from the National Center for Biotechnology Information database, using the CLC sequence viewer software, version 7.8.1. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with blue residues being the least conserved and white residues being the most conserved. Besides, a yellow-colored plot in percent scale shows the conservation extent of each domain

in three different autotrophic, heterotrophic, and mixotrophic growth conditions. As depicted in Figure 3, the first 3 days were considered as the lag phase period (0.295×10^6 cell mL^{-1} in autotrophic, 0.292 x 10^6 cell mL^{-1} in heterotrophic, and 0.290×10^6 cell mL^{-1} in mixotrophic modes). After it, the logarithmic growth phase was experienced in the 3rd–15th day of experiment. The all three studied cultivation modes entered to the stationary phase at the 15th day of study 2.128×10^6 cell mL^{-1} in autotrophic, 1.540×10^6 cell mL^{-1} in heterotrophic, and 3.322×10^6 cell mL^{-1} in mixotrophic modes). At the end of the stationary growth phase (21st day), the final cell numbers of 3.116×10^6 cell mL^{-1}, 2.456×10^6 cell mL^{-1}, and 5.050×10^6 cell mL^{-1} were found in autotrophic, heterotrophic, and mixotrophic modes of cultivation, respectively. The results of cell dried weight measurement were in quite agreement with the results of direct cell counting method. On the other words, at the end of the 3rd day, cell dry weight amounts were reached to 0.24, 0.16, and 0.22 g L^{-1}, respectively. At the end of logarithmic phase (15th day), the measured biomass reached up to 1.79 g L^{-1}, 1.04 g L^{-1}, and 2.51 g L^{-1}, respectively. At the end of the stationary phase, the final cell dry weight of the studied strain was found to be 2.47 g L^{-1} in autotrophic, 1.52 g L^{-1} in heterotrophic, and 3.91 g L^{-1} in mixotrophic growth condition.

Biomass Composition Analysis

The final lipid content of *C. vulgaris* was found to be 0.84 g L^{-1} (34.01% in the total obtained biomass) in autotrophic, 0.74 g L^{-1} (48.68%) in heterotrophic, and 1.73 g L^{-1} (44.25%) in mixotrophic growth condition [Figure 4]. On the other words, after 21 days of microalgal growth, the obtained lipid amount in heterotrophic mode was elevated up to 143.13% and 110.01% in comparison with autotrophic and mixotrophic mode, respectively. Besides, the ultimate level of lipids in mixotrophic culture was found to be 130.11% higher than the autotrophic mode. The obtained lipids could be exploited for food or feed application and also for biodiesel production.

The total concentrations of protein and carbohydrates in the final biomass were also investigated. It was revealed that the ultimate levels of the proteins and carbohydrates in the microalgal cells grown in autotrophic medium to be 1.03 g g^{-1} (41.702%) and 0.43 g g^{-1} (17.41%), respectively [Figure 4a]. Moreover, a cumulative amount of 0.17 g g^{-1} comprising the 6.88% of the final biomass (2.47 g L^{-1}) was considered as nucleic acids, other impurities, and probable errors. The results of the same experiments in heterotrophic culture medium revealed that the final protein and carbohydrate concentration at the end of study was 0.51 g g^{-1} (33.55%) and 0.20 g g^{-1} (13.16%), respectively [Figure 4b]. The quantity of remained ingredients and compounds was 0.07 g g^{-1} that is regarded to be 4.61% of the final attained biomass (1.52 g L^{-1}). Furthermore, in mixotrophic condition, the maximum levels of proteins and carbohydrates were found to be 1.73 g g^{-1} (31.20%) and 1.22 g g^{-1} (19.44%), namely. Notably, 0.20 g g^{-1} (5.11%) of the remaining materials were considered as nucleic acids and impurities while the ultimate amount of microalgal biomass was detected as 3.91 g L^{-1} [Figure 4c].

DISCUSSION

The results of morphologic and molecular methods confirmed the identification of *C. vulgaris* strain. It could be determined that the maximum amounts of cell numbers and dry weights were observed in mixotrophic growth mode. The biomass production amounts in mixotrophic mode were increased 158.30% and 257.24% in 21 days compared with autotrophic and heterotrophic conditions, respectively. Moreover, the observed levels in autotrophic growth condition were higher (162.50%) than the heterotrophic study. The maximum amounts of proteins were found in autotrophic mode while the mixotrophic growth condition leads to the maximum amounts of carbohydrate production. The observed changes in *C. vulgaris* biomass ingredients revealed the variations of microalgal cell metabolism and physiology during three different studied conditions. Furthermore, based on the biomass composition data, the isolated *Chlorella salina* strain might be considered as a robust platform for biomass, lipid, and protein production. Each studied cultivation approach could be employed to achieve higher contents of lipids (heterotrophic), proteins (autotrophic), and carbohydrates (mixotrophic).

Mohammad Mirzaei *et al.*[20] have achieved the maximal dry weight of 2.62 g L^{-1} composed of 0.86 g L^{-1} lipids in mixotrophic cultivation of *C. vulgaris* on agricultural waste medium which stands for 140% (biomass) and 170% (lipids) increase compared with autotrophic and 300% (biomass) and 1200% (lipids) heterotrophic modes of cultivation, respectively. In another study, Li *et al.*[21] have shown 1.74, 14, and 5.6 times' increase in the specific growth rate, biomass production, and lipid productivity, respectively, with mixotrophic growth compared to the autotrophic growth. The obtained results are quite in agreement with our findings which mixotrophic cultivation mode achieved the maximal amounts of biomass and lipid in the studied *C. vulgaris* strain. Moreover, in another study, it has been revealed that *Chlorella pyrenoidosa* could be cultivated in mixotrophic mode using wastewaters to obtain a cost-effective and feasible alternative commercial medium for microalgal biomass production without the need for addition of an expensive organic carbon source to the culture medium.[22] On the other hand, heterotrophic mode of growth is regarded as the best cultivation strategy for lipid accumulation.[10] In the current study, we also observed the maximal lipid aggregation of 48.68% in heterotrophic metabolism. Although these results might be incompatible in some cases due to possible variations in culture media characteristics such as the glucose

concentration, nitrogen source and concentration, CO2 concentration, and also the light intensity.[23]

This study confirmed the possibility of growing *C. vulgaris* on glucose as the carbon source to achieve higher levels of lipids in comparison with the autotrophic or mixotrophic methods of cultivation. Besides, the studied strain would grow under mixotrophic conditions which allows its broad uses in various applications and also to achieve higher cell densities. The observed phenomenon is attributed to the carbon availability for the microalgal cells which causes the increased proportion of storage lipids.[24] Finally, considering the higher levels of lipids, produced in heterotrophic condition in the strain, this culture is recommended for lipid production which can be used to produce biofuel and also polyunsaturated fatty acids, as the food supplements.

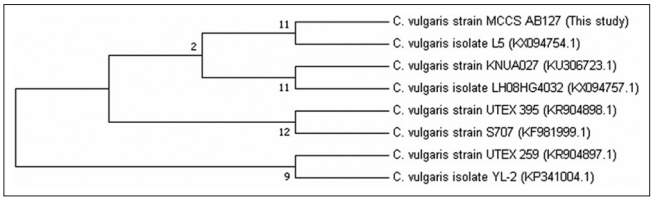

Figure 2: Molecular phylogenetic analysis by maximum likelihood method based on 18S rRNA sequences of eight *Chlorella vulgaris* strains. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the analyzed taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Scale bar represents 0.0005 substitutions per nucleotide position

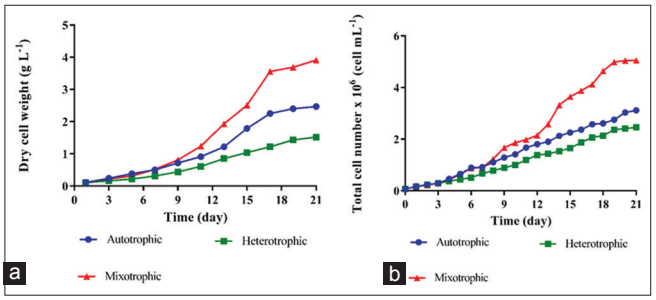

Figure 3: The effects of autotrophic, heterotrophic, and mixotrophic modes of cultivation on growth trend of *Chlorella vulgaris* using (a) dry cell weight (g L−1) and (b) direct cell counting (cell mL−1) methods

CONCLUSION

The influences of three different cultivation conditions including autotrophic, heterotrophic, and mixotrophic modes of cultivation on growth pattern, biomass production, and composition in a naturally isolated *C. vulgaris* strain were investigated. Under autotrophic and mixotrophic cultivation modes, the appropriate amounts of proteins and carbohydrates were achieved while the heterotrophic mode was the best for the lipid production. The studied microalgal strain was confirmed as a robust candidate for biomass, lipid, and protein production for different pharmaceutical, nutritional, and industrial purposes.

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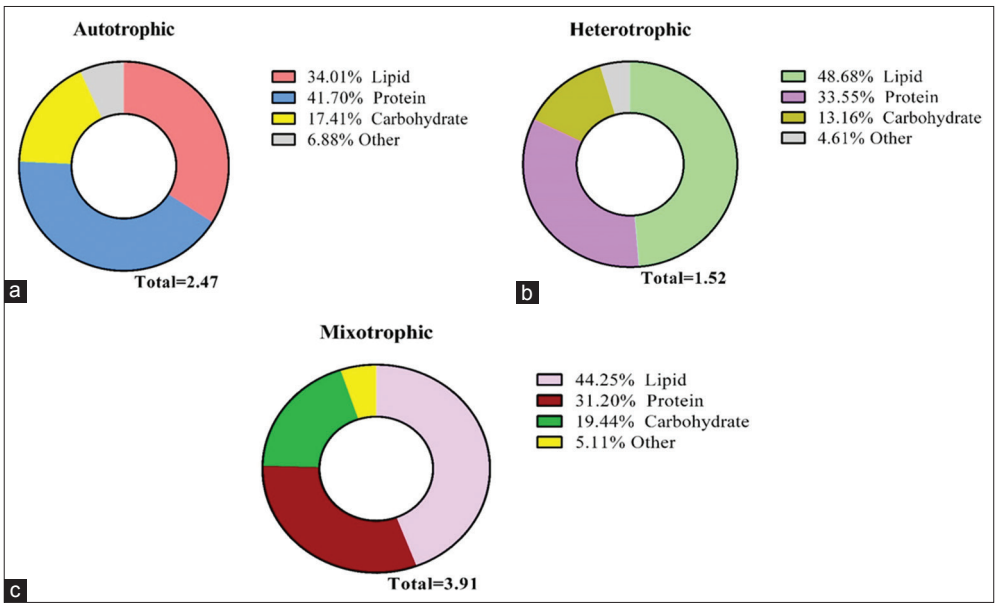

Figure 4: Biomass production and composition analysis including the total lipids, proteins, and carbohydrate in (a) autotrophic, (b) heterotrophic, and (c) mixotrophic growth conditions

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