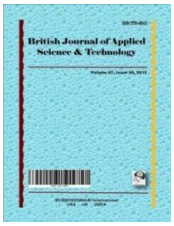

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Influence of Temperature and CO_2 on the Growth and Accumulation Oil of Microalgae

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Strains of algae belonging to the genera *Scenedesmus*, *Stichococcus*, *Chlorococcum*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Coelastrum,* and *Pediastrum* were isolated from water samples collected in Uzbekistan. At optimum temperature (22ºC, 24ºC, 28ºC), all cultures were mesophilic algae. The results show that for these microalgae, 2% carbon dioxide is sufficient for optimal growth and development. The maximum accumulation of lipids (46,6-55,0%) was observed in cell cultures *Chlorococcum* sp.4, *Chlorococcum* sp.8, and *Chlorococcum* sp.37. In oils of *Ankistrodesmus angustus,* UT-15 detected 7 fatty acids, whereas in *Pediastrum* sp.1, 16 fatty acids were detected.

Keywords: Microalgae; oil; fatty acid.

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1. INTRODUCTION

Temperature is an important environmental factor that affects the livelihood of algae, which, in contrast to other elements of the environment, cannot be excluded from an organism's environment. In the growth and development of each species and strain of microalgae, the minimum, optimum, and maximum temperature can be determined. Thus, even small changes in temperature may change the rate of metabolic reactions and the overall intensity of an exchange. There are microalgae strains resistant to low temperatures (cryophilic), as well as strains and thermophilic adapted to relatively high temperatures (above 40ºC) [1].

Algae that thrive in average temperatures (15-30ºC) are called mesophilic. *Cryophilic* algae, on the other hand, grow slowly, due to their low productivity. For example, the brown alga *Phaeocystis poucheti* has a temperature range of -1ºC to + 11ºC. However, algae have adapted to a wide temperature range, within which their growth and reproduction is possible [2]. The algae eurythermal species are able to tolerate high fluctuations in temperature e.g., the diatom *Nitzschia putrida* has a temperature regime equal to 11- 42ºC [3]. Other algae, such as stenothermal, develop in a narrow temperature range. Temperature plays a key role in photoinhibition, known to affect the growth rate of algae [4].

Increases in concentration of unsaturated fatty acids in the lipid layer of microalgae cell membranes are often observed with temperature change [5,6]. A significant increase in the unsaturation of fatty acids occurs in response to a decrease in temperature from 30ºC to 12ºC [7]. The lower temperature reduces the fluidity in the cell membrane. The cells compensate by raising the level of unsaturated fatty acids to increase membrane fluidity. Nevertheless, the membrane becomes more susceptible to free radical damage [8,9]. The lipid content doubles as the temperature increases from 20ºC to 25ºC (from 7.90% to 14.92%) in the *N. oculata* [10]. An increase in temperature beyond the optimum reduces protein synthesis and thus leads to a decrease in the growth rate [11]. Mesophilic algae are most suitable for the production of algae in open settings. They are highly adaptable to temperature conditions, grow well, and develop in spring and autumn temperatures and daylight conditions [12].

Intensive use of natural energy sources has led to an increase in the concentration of carbon dioxide in the atmosphere the main cause of global warming. One of the primary ways of reducing CO_2 in the atmosphere is biological fixation via plants. Microalgae more efficiently capture CO_2 and can grow about 10 to 50 times faster than terrestrial plants [13].

Microalgae convert atmospheric CO_2 to polar and neutral lipids, and can therefore be utilized as an alternative fuel through esterification. Microalgae capture solar energy more efficiently than the conventional oleaginous plants under nutritional limitation and stress conditions [14,15]. Biomass concentration, increasing lipid content, and overall lipid productivity determine the economic feasibility of algal oil for biodiesel production. Therefore, process optimization that can maneuver the algal biochemical production, fast growth, and good environmental adaptability helps to achieve environmental and economic sustainability. An ideal process should produce the highest productivity of algae with enhanced cellular lipid content.

Based on the above stated objective, the present study aimed to determine the optimum conditions for growing microalgae isolated from different regions of Uzbekistan and to study accumulation oil in the cells of microalgae.

2. MATERIALS AND METHODS

2.1 Strains of Microalgae

The objects of study used were strains of unicellular green algae genera *Scenedesmus*, *Stichococcus*, *Chlorococcum*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Coelastrum* and *Pediastrum,* isolated from water sources in Uzbekistan [16].

2.2 Growth Condition

Cultures of microalgae were grown under sterile conditions in modified "Chu- 13" medium for 14 days at 22ºC, 24ºC, 28ºC, with a supply of carbon dioxide by blowing air containing 2% CO_2 and with continuous illumination by fluorescent white light (200 μmol photons $m^{-2} \cdot s^{-1}$).

2.3 Lipid Extraction

Extraction and determination of intracellular lipids of microalgae was conducted according to standard protocols outlined in the previous literature [17]. Dry biomass of algae was placed in a glass mortar and sand was added in the ratio

1:4 i.e., 1 portion of plant biomass to 4 portions of glass sand and comminuted until smooth. Methanol, DMSO (volume fraction) and a mixture hexane and diethyl ether (1:1, volume ratio) was added to the biomass and stirred for 1 hour at room temperature (24ºC). Then the mixture was centrifuged (3000 g 10 min), and the upper phase was collected and transferred to a round bottom flask. The residua were re-extracted 3 times with a mixture hexane and diethyl ether for 30 min. Each time, the mixture was shaken and then centrifuged for 10 min at 3000 g and the upper organic layer was collected. All organic phases were combined, evaporated to dryness, were placed under nitrogen and then weighed.

2.4 Fatty Acid Analysis

To determine their fatty acid composition, each sample of lipid was hydrolysed by 10% methanolic KOH solution in the ratio of 1:10 (sample:solution) in a boiling water bath for 1 hour. The resulting soap was decomposed with 50% aqueous H_2SO_4. Fatty acids were extracted three times with diethyl ether. Next, ether extracts were washed with distilled water to neutralize pH, dried over sodium sulfate, and then ether was distilled off. The fatty acids were methylated with newly prepared diazomethane. Purification of the resulting methyl ester was carried out in a thin layer of silica gel using the solvent system hexane: diethyl ether 4:1. Fatty acids were determined with the instrument Agilent Technologies 6890 N, with a flame ionization detector using a capillary column 30 m long with an internal diameter of 0.32 mm and the applied phase HP-5 at a temperature of 150 to 270ºC with helium as the carrier gas.

2.5 Reagents

Silica gel used in purification of oil of algae was purchased from Sigma-Aldrich. All other reagents were from Ximbiogen (Uzbekistan).

3. RESULTS AND DISCUSSION

3.1 Isolation of Microalgae

Accumulative cultures of microalgae were obtained from water samples collected in Uzbekistan. The cultures differed in slurry density and uniformity. Using the dilution method, the samples of accumulative cultures were sowed to the 2% agar modified Chu 13 medium. Within 7 days of incubation, very small, individual colonies of microalgae appeared on the surface of the agar medium, and after three weeks, colony size reached 2-3 mm. Subsequently, individual colonies of microalgae were grown in a liquid medium for 7 - 15 days, depending on the growth rate of cultures.

Thus, we isolated and purified local unicellular green algae belonging to the genera *Scenedesmus*, *Stichococcus*, *Chlorococcum*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Coelastrum,* and *Pediastrum* from aqueous samples from Uzbekistan.

3.2 Effect of Temperature on Algae Growth

The goal in the first stage of the study was to optimize the growth conditions of microalgae. In this regard, we have begun to study the effects of temperature on the growth and development of microalgae.

When beginning the cultivation of strains of *Scenedesmus quadricauda* UT4 and *Scenedesmus* sp.25, we observed similar growth and development of the microalgae regardless of the temperature (Figs. 1a, b). As the results show, when the temperature was changed between 22ºC, 24ºC, and 28ºC, the growth speed of *Scenedesmus* strains varied. During the 14 day cultivation, a maximum biomass of microalgae (123-130 mg/L^{-1}) was observed at the temperature of 24ºC, compared to 22ºC and 28ºC. The oil content in the cells of cultures was 36% - 41% of the total biomass of the microalgae.

The temperature for growing microalgae *Stichococcus* sp.1 was strictly kept at 22ºC, the optimum temperature, and the oil content was 44.5%.Under various temperatures (22ºC, 24ºC, 28ºC), *Coelastrum* sp.1 swiftly developed, and the color of suspension was light green. When increasing temperature from 22ºC to 28ºC, the biomass of the study cultures regularly increased from 268 mg /L^{-1} to 395 mg /L^{-1} (Fig. 1 a). A high content of oil (38.5%) in *Coelastrum* sp.1 cells was detected at 28ºC (Fig. 1 b).

On the tenth day of cultivation, the density and color of the suspension of microalgae strains *Chlorococcum* significantly differed from one another, particularly *Chlorococcum* sp.37, which had a darker green color compared to *Chlorococcum* sp.4 and *Chlorococcum* sp.8. Upon further culturing (14 days), *Chlorococcum* sp.4 and *Chlorococcum* sp.8 suspension of microalgae took on a yellowish shade.

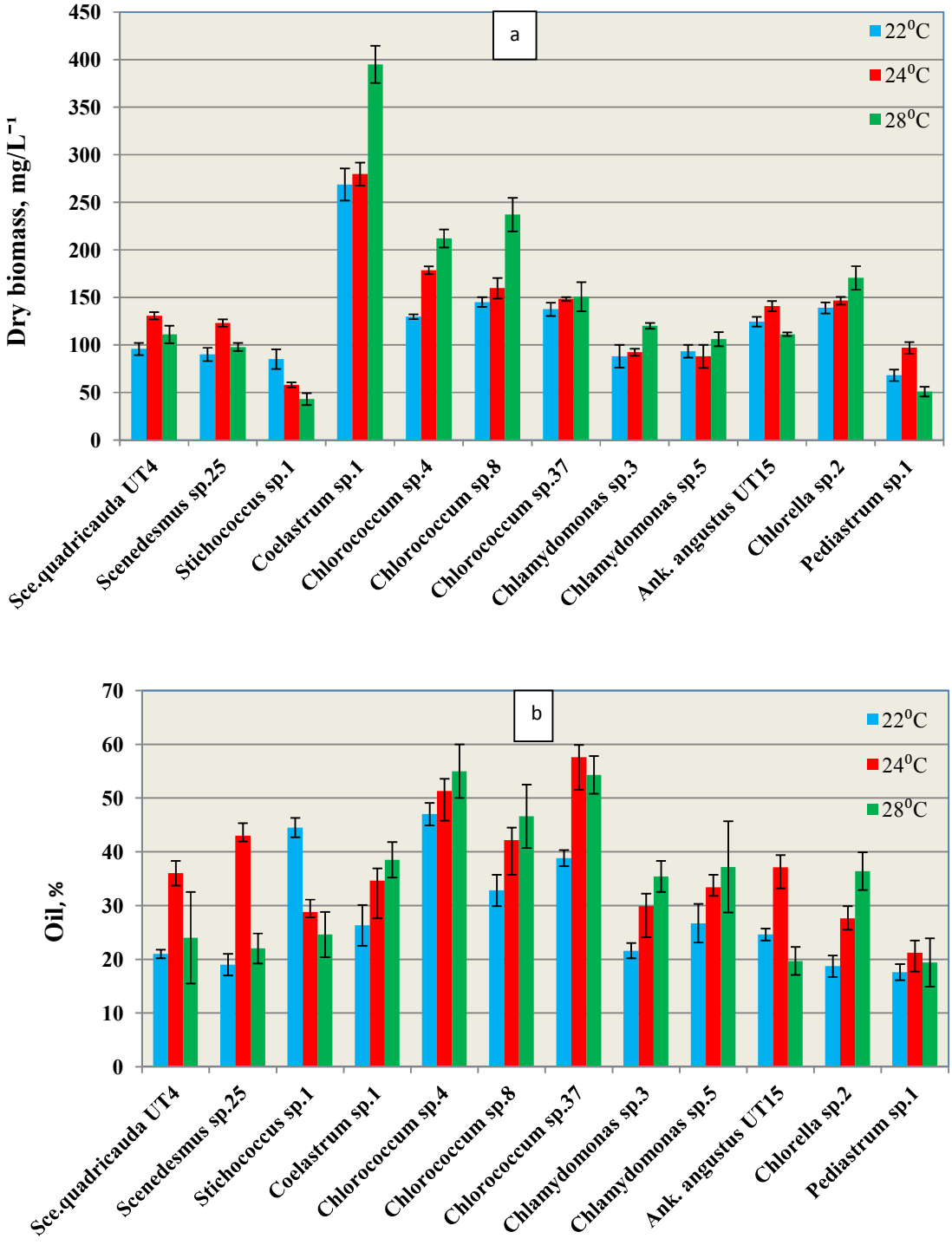

Fig. 1. Effect of temperature on the content of biomass (a) and oil (b) of microalgae, grown for 14 days on a modified Chu 13 medium. Standard deviations obtained from triplicate determinations

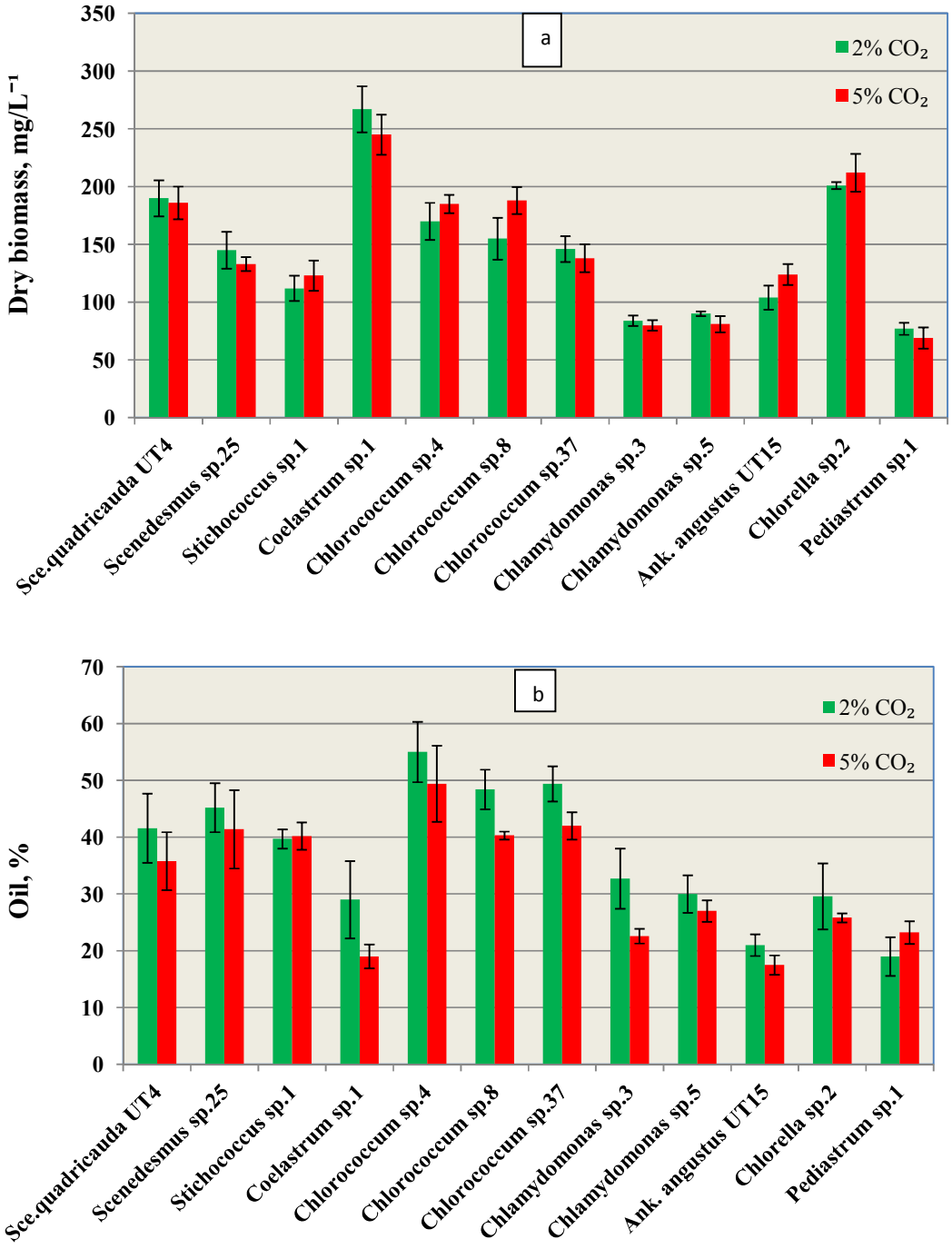

Fig. 2. Effect of CO2 on the content of biomass (a) and oil (b) of microalgae, grown for 14 days on a modified Chu 13 medium. Standard deviations obtained from triplicate determinations

When culturing microalgae at 22ºC temperature, the range of biomass accumulation was 129 mg/L^{-1} to 145 mg/L^{-1} (Fig. 1a). The highest accumulation of biomass (150-237 mg/L^{-1}) and oil (46.6-55.0%) was achieved at 28ºC (Figs. 1a, b). The direct correlation between the accumulation of biomass and oil was found in

strains *Chlorococcum* sp.4 and *Chlorococcum* sp.8.

Chlamydomonas strains formed a biomass from 88 to 93 mg /L^{-1} at 22ºC, and 106-120 mg /L^{-1} at 28ºC (Fig. 1a). The maximum accumulation of oil (27-37%) was observed at 28ºC.

Studying the effect of temperature on the growth and development of the strain *Ankistrodesmus angustus* UT15, it was found that at 22ºC, 24ºC, and 28ºC the culture grew well and formed a relatively similar biomass at each temperature. The optimum growth temperature for the strain *Ankistrodesmus angustus* UT15 was 24ºC. Maximum oil accumulation was 37.1% (Fig. 1b).

The optimum temperature for the strain *Chlorella* sp.2 was 28ºC, and this strain formed and oil accumulation of 36.4% (Fig. 1b).

For the strains of the genus *Pediastrum,* the optimum temperature was 24ºC. Increasing the temperature to 28ºC resulted in a decrease of biomass (Fig. 1a).

Based on the data obtained, we can conclude that all of the local strains of microalgae, depending on the growth temperature, were mesophilic microalgae and had the following optimum temperatures: *Stichococcus* - 22ºC; *Ankistrodesmus* - 24ºC; *Pediastrum* - 24ºC; *Scenedesmus* - 24ºC; *Chlorococcum* - 28ºC; *Chlamydomonas* - 28ºC; *Coelastrum* - 28ºC; *Chlorella* - 28ºC.

3.3 Impact of CO_2 on Algae Development

The growth and productivity of microalgae was studied in a neutral medium of pH 7.0 with different concentrations of carbon dioxide (CO_2) at 28°C. Microalgae grew well and accumulated similar biomass regardless of CO_2 concentration (2%, 5%) (above Fig. 2 a). At 2% CO_2, the strain of *Coelastrum* sp.1 formed large biomass (237 mg/L^{-1}) in comparison with the other studied cultures (Fig. 2a). When cultures were grown under 2% CO_2, the maximum accumulation of oil (45.2-55%) was found in the cells of cultures *Chlorococcum* sp.4, *Chlorococcum* sp.8, *Chlorococcum* sp.37, and *Scenedesmus* sp. 25 (above Fig. 2b). It should be noted that lipid accumulation was slightly higher in microalgae grown in 2% CO_2 compared to 5% CO_2, except for strains *Stichococcus* and *Pediastrum*. These results indicate that for these microalgae, 2% CO_2 is sufficient for optimal growth and development. A high yield of microalgae biomass (about 1 g per 1 liter/h) was prepared at a concentration of 0.8% CO_2, which was increased to 5.8% CO_2 did not affect the yield of microalgae. The CO_2 was then decreased to 0.2%, which reduced the efficiency strain of *Chlorella* 2 times [18]. Chiu et al. showed that CO_2 above 5% may be harmful to microalgal cells and inhibit the growth of Nannochloropsis oculata [19]. In contrast, microalgae like S. obliquus can grow beyond 6% CO_2 and reach a maximum biomass at 18% CO_2 [20].

3.4 Fatty Acid Profile of Selected Algae

To determine the qualitative and quantitative composition of fatty acids, we selected microalgae strains *Ankistrodesmus angustus* UT15, *Coelastrum* sp.1, *Chlorella* sp.2, *Scenedesmus* sp.25, *Chlorococcum* sp.4, and *Pediastrum* sp.1.

After cultivation of these microalgae strains for 14 days, 18 fatty acids were found in the cells of the algae (decanoic, dodecanoic, tetradecanoic, pentadecanoic, hexadecenoic, palmitic, heptadecanoic, gamma-octadecatrienoic, stearidonic, linoleic, oleic alpha-linolenic, stearic, eicosenoic, eicosanoic, docosanoic, tetracosanoic, *hexacosanoic* acids).

In the cultures of *Ankistrodesmus angustus,* UT15 detected 7 fatty acids, whereas in the strain *Pediastrum* sp.1, 16 fatty acids were detected. Despite the presence of different fatty acids in the microalgae, the most common acids among the saturated fatty acids were palmitic acid (C16:0, at 26.65% to 44.96%) and stearic acid (C18:0, at 3.07 % to 5.67%) (Fig. 3). Among unsaturated acids predominant oleic (C18:1) and alpha-linolenic (C18:3) acids makes up from 16.8% to 43.75%. The proportion of linoleic acid (18:2) was in the range of 8.53-35.86%.

It must be emphasized that biodiesel derived from saturated fatty acids (palmitic and stearic acids) has a higher cetane number and is of higher quality [21], whereas esters from unsaturated fatty acids show lower cetane numbers and are susceptible to oxidation [22].

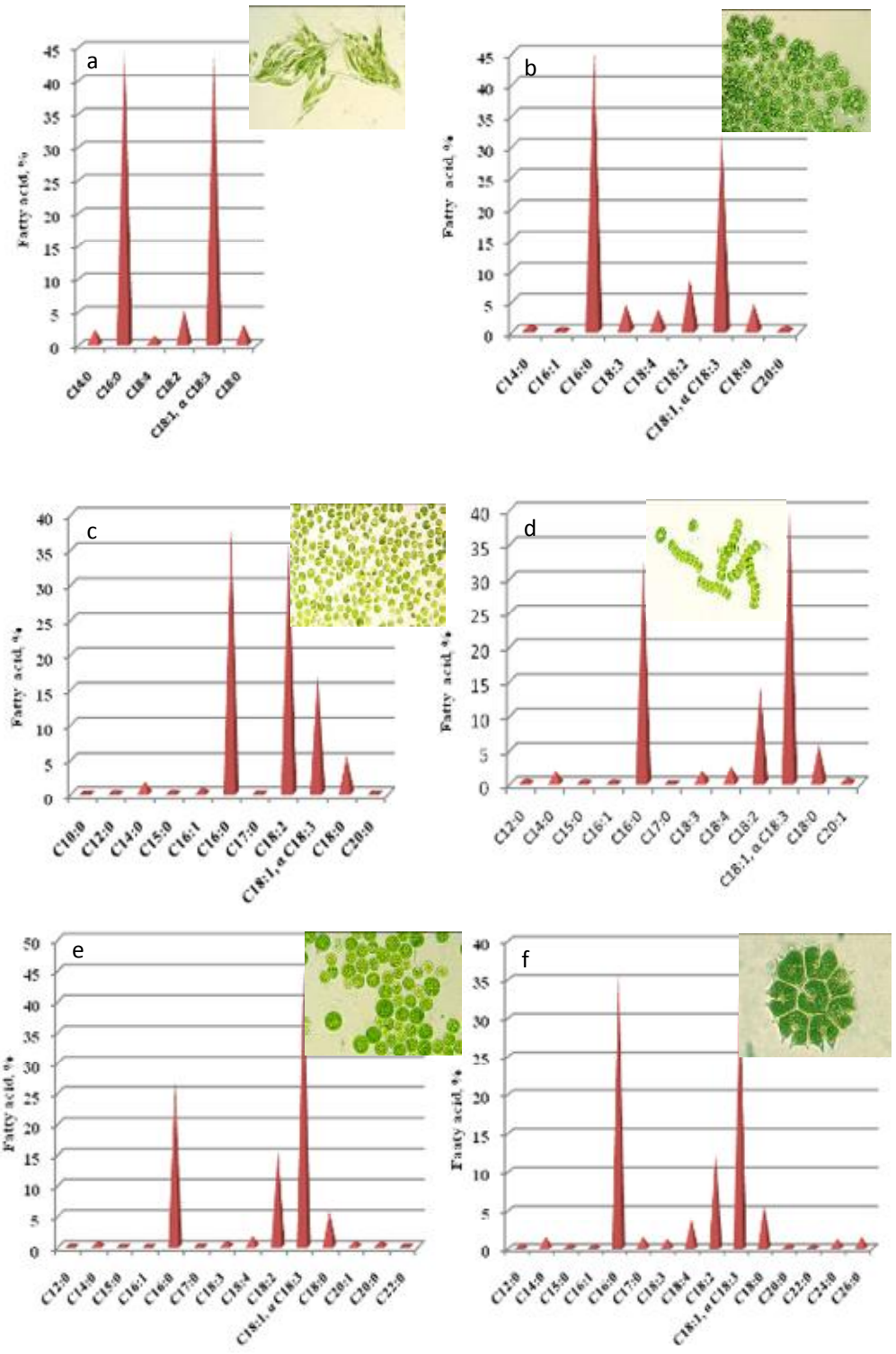

Fig. 3. Main fatty acids present in microalgae: *Ankistrodesmus angustus* UT15 (a), *Coelastrum* sp.1 (b), Chlorella sp.2 (c), *Scenedesmus* sp. 25 (d), *Chlorococcum* sp.4 (e) and *Pediastrum* sp.1 (f)

In the cells of *Pediastrum* sp.1, *Ankistrodesmus angustus* UT15 and *Coelastrum* sp.1's saturated fatty acid content is 49.53% to 51.18% of the total fatty acids. In all of the studied microalgae, up to 97% of the fatty acids are composed of palmitic, stearic, linoleic, oleic, and alpha-linolenic acids.

4. CONCLUSION

Microalgae isolated from water sources of Uzbekistan accumulated high amounts of biomass and oil in respective optimum temperatures and CO2 levels. In addition, the composition and amount of fatty acids depended on genus and species of microalgae. Due to the formation of biomass and accumulation of lipids and fatty acid composition, the cultures *Scenedesmus quadricauda* UT4, *Coelastrum* sp.1, *Scenedesmus* sp.25, *Stichococcus* sp.1, *Chlorococcum* sp.4, *Chlorococcum* sp.8, *Chlorococcum* sp.37, and *Chlorella* sp.2 may be used as potential producers of oils for biodiesel production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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