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Bioprospecting of Ten Microalgae Species Isolated from Saline Water Lake for Evaluation of the Biodiesel Production

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Abstract

Algal bioprospecting in ecosystems leads to exploring native microalgae and the competency evaluation of economically producing lipids as biofuel or nutritional applications. In this study, ten microalgae species were screened from the saltwater lake. *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlamydomonas raudensis*, *Chlamydomonas hedleyi*, *Dunaliella salina*, *Picochlorum bazangan* sp. nov., *Tetraselmis bazangan* sp. nov., *Haematococcus lacustris*, *Nannochloropsis oceanic*, and *Scenedesmu rubescens* were isolated and identified using 18SrDNA and *tuf*A markers. Biodiesel potentials were assayed by the determination of biomass productivity, biochemical components, fatty acid profile, and biodiesel properties. The results showed that the maximum biomass yield (1.22 gL^{-1}) belonged to *C. vulgaris*. The highest protein, carbohydrate, chlorophyll, and carotenoid content were recorded in *C. vulgaris*, *C. raudensis*, *C. sorokiniana*, and *D. salina*, respectively. *N. oceanica* accumulated high lipid content and omega-3 fractions (31.09%). However, *C. hedleyi* had the highest lipid productivity (11.64 $gL^{-1}d^{-1}$) compared to other microalgae. The best species for biodiesel production was *C. vulgaris*, with a specific growth rate of $0.36d^{-1}$, lipid productivity of 7.45 $gL^{-1}d^{-1}$, and C16-C18 fatty acid profile of 78.3%. The microalgae *C. vulgaris* had appropriate biodiesel properties of low viscosity (4.49), high cetane number (55.38), and relatively low cloud point (4.98). Another choice was *N. oceanic*, with high lipid productivity, cetane number (59.79), oxidative stability (56.43), and low iodine value (47.11). Microalgae *T. bazangan* sp. nov. had a cetane number (55.24), low cloud point (4.71), and C16-C18 fatty acid profile of 82.34%. Accordingly, *C. vulgaris*, *T. bazangan* sp. nov., and *N. oceanic* can be considered potential species for biodiesel.

Keywords Biodiesel properties. Bioprospecting. Fatty acid profile. Lipid content. Microalgae.

Introduction

Global crude oil consumption has increased in the last half-century due to rapid industrialization and population growth [1]. The usage of these fuels has destructive effects such as global warming, ecological degradation, and health issues [2]. The first and second generations of biofuels were derived from food sources and cellulose derivatives [3, 4]. However, the biofuels derived from soybean (*Glycine max*) and canola (*Brassica napus*) have several disadvantages, for example high prices and increased greenhouse gas emission [5, 6]. Lipid-rich microalgae can be used for biofuel and especially biodiesel production due to their lipid yield being higher than those of the best oilseed crops [7]. Microalgae can be considered an alternative candidate for biofuel production and the third generation of biofuels [8, 9].

Microalgae are a highly diverse group found in different environments, such as freshwater and marine systems. They are very efficient in collecting sunlight and converting it into biochemical compounds, which causes them to have a higher production rate than plants [10, 11]. The high percentage of valuable compounds such as proteins, carbohydrates, pigment, lipids, minerals, antioxidants, and vitamins in microalgae makes them an excellent source for different industries, including medicine, cosmetics, food, feed, and biofuel [12]. Some algae are a good selection for biodiesel production due to their high growth rate and ability to store lipids [13]. The lipid of algae is divided into three groups according to lack, presence, and number of double bonds. Saturated fatty acids (SFAs) with no double bond in the hydrocarbon chain, whereas monounsaturated fatty acids (MUFAs) have one double bond per molecule, and Polyunsaturated fatty acids (PUFAs) with several bonds in the hydrocarbon chain (in polar or neutral forms) [14]. Microalgae produce different lipid content (between 20% and 70% of their fresh weight) depending on the species and environmental conditions [15].

The fatty acid composition has gained special attention due to its health benefits for consumers [13]. In addition to using algae biomass as fertilizer and food, biofuel produced from algal oil is an important carbon-free renewable energy source and an efficient means of carbon sequestration because has carbon neutral, and burning it does not increase the net carbon content of the atmosphere [13]. On the other hand, the cultivation of algal biomass does not require arable land, they can even grow in urban, agricultural, and industrial wastewaters that have nutrients [16].

Bioprospecting of algae is defined as the identification of economically valuable biochemical resources from algae rich in such content and enables industrial bioresource generation [13]. The biomass of many species of microalgae is a rich source of proteins, lipids, pigments, and carbohydrates that algal products manufacture and commercialization can benefit society [13]. The few species of microalgae currently used in the industry are insufficient to meet the existing demand for the same. Exploration of native microalgae and evaluation of the competency of biochemical content is highly significant. Moreover, native algal species are generally adapted to a wide range of environments [17]. The choice of an appropriate microalgae species with high biomass and lipids content is essential to reduce the time and cost of producing biodiesel, especially for large-scale cultivation [18]. Selvarajan et al. reported that *C. vulgaris* LC8 was the best species for biofuel production compared to other microalgae species isolated from freshwater and soda Lakes [19]. In the studies of Abou-Shanab et al. and Pereira et al., *Scenedesmus sp.* and *Tetraselmis sp.* CTP4 introduced lipid-rich microalgae, which are commonly used for biodiesel production [20, 21]. Moreover, in some research new species of microalgae with high oil and protein levels are investigated [22, 23].

Bazangan Lake is located in Sarakhs, Khorasan-Razavi Province, Iran, with an average annual temperature of 22˚C. So far, no report of isolated microalgae from saline water Lake (Bazangan) in Iran has published. This report is the first screening of microalgae species isolated from this Lake for biofuel production.

The major aim of the present study was identified by the exact taxonomy of the eight isolated as the common and dominant species from Bazangan Lake by molecular characterization using 18SrRNA and *tufA* markers. The other goal were to research biomass yield, carbohydrate, total protein, pigment content, total lipid content, lipid productivity of the same algae. The fatty acid profiles of microalgae in SFA, PUFA, and MUFA also assayed. Moreover, biofuel properties of microalgae species such as average degree of un-saturation (ADU), kinematic viscosity (KV), cloud point (CP), specific gravity (SG), cetane number (CN), iodine value (IV), higher heating value (HHV), oxidative stability (OS), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP) were examined to find the main application potential in different industries.

Materials and methods

Sampling site

Bazangan Lake (36°.19′ N, 60°.29′ E) with an area of 80 hectares is located in Sarakhs, Khorasan-Razavi Province, Iran, at an altitude of 850 m above sea level. The sampling was performed from eight different sites of Bazangan Lake, in the period April 2019 to May 2020 (Fig. 1). 500 ml of water were randomly collected in a sterile glass bottles, and maintained at 4°C.

Cultivation and purification of microalgae

About 100 milliliters of the water from each sample was centrifuged at 4500 ×g for 4 min. To isolate microalgae, the dilution plating method was used according to Robert [24]. One milliliter of water containing concentrated algae cells was cultured in a sterile medium prepared with the Lake water and spread across the surface using a glass l spreader. Plates were inoculated in a controlled culture room at 25 ± 2 °C and irradiance of 100 µmol photons m^{-2} s^{-1} with 16:8 h light: dark cycle. After the microalgae growth for five weeks, single colonies were streaked separately on plates and maintained under the same conditions mentioned above. Transfer of microalgae colonies and the streaking method were repeated until a pure isolate culture was obtained. Following the isolation of microalgae, each species was cultured on LB. After confirming the purity of isolates, microalgae species were identified with 18SrDNA and *tuf*A barcode genes.

Molecular identification and phylogenetic analysis

Genomic DNA was extracted from the microalgae isolates using a Plant and Tissue kit (Sepidanazma, Iran) according to the manufacturer's instructions. The concentration and purity of DNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and standard electrophoresis, respectively. The *tuf*A and 18SrDNA barcode regions were amplified with the primers tufAGF4 (GGNGCNGCNCAAAT GGAYGG), tuf_R (CCTTCNCGAATMGCRAAWCGC) [25] and 18S-F (GTCAGAGGTGAAATTCTTGGAT TTA), 18S-R (AGGGCAGGGACGTAATCAACG) [26]. Moreover, PCR reactions were carried out in a thermal cycler (BioRad Dyad, USA). PCR program for *tuf*A and 18SrDNA regions were 30 cycles of denaturation at 94°C for 45s and 30s, annealing at 60°C for 45s and 30s and elongation at 72°C for 60s and 45s, respectively. The products were confirmed by 1% agarose gel electrophoresis and then sent to the Macrogen Company (South Korea) for bidirectional sequencing.

The sequences were edited with CLC Sequence Viewer-6-8-1 software. The sequences were compared with related taxa from the GenBank database at the NCBI using the Blast program [27]. For phylogenetic analysis,

sequences were aligned with other sequences using MEGA 7.0.26 software [28]. The phylogenetic trees were constructed using the neighbor-joining (NJ) method and Kimura's 2-parameter (K2P) distance model as recommended by Hebert with bootstrap values of 1000 replicates [29].

Growth measurement and biomass dry weight

The microalgae were cultured in the optimal medium (Table 4), under the same condition mentioned above, for 16 days (the stationary growth stage). The specific growth rate (SGR) of species was calculated according to the following equation, as specified by Wood et al. [30]:

$$\mu = \frac{(\mathrm{LnNf} - \mathrm{LnN0})}{\mathrm{tf} - \mathrm{t0}}$$

Where μ (d^{-1}) is the specific growth rate during the stationary growth phase, t_f and t_0 are the final and initial time, respectively and N_f and N_0 are the optical density (at 680 nm using a Nanodrop (S2100SUV)) at time t_f and t_0, respectively.

For biomass yield calculation, the microalgae cells per liter were harvested via centrifugation (8000 rpm for 10 min) in the late stationary growth stage and then dried in a freeze-drier (VaCo5, Germany) to a constant weight. Biomass productivity was determined via the below equation [31]:

$$\text{Biomass productivity} = \text{biomass yield} \times \mu$$

Biomass productivity (g L^{-1} d^{-1}) is the dry biomass produced in the stationary growth stage, biomass yield (g L^{-1}) is the yield into biomass, and μ (d^{-1}) is the specific growth rate.

Analyses of biochemical components

For the determination of protein, lipid, carbohydrate, and pigments percentage in microalgae species, the dried biomass was extracted by standard methods. The total protein percentage was determined spectrophotometrically by the Folin phenol reagent according to the Hartree method [32]. For this purpose, 8 mL of 0.5 M NaOH was added to 10 mg microalgae biomass and incubated at 80 °C for 10 min. After the centrifuge, 0.5 mL of 0.5 M sodium carbonate, 0.5 mL of 0.5 M sodium potassium tartrate, 0.5 mL of 0.5 M copper sulfate, and 2 mL of Folin-Ciocalteu reagent were added to the supernatant. The calibration curve was obtained with different concentrations of BSA (Bovine Serum Albumin).

Total lipid was extracted from approximately 100 mg of microalgae powder using a modified method of Bligh and Dyer [33]. The harvested cells were extracted using a mixture of chloroform: methanol: and distilled water at a ratio of 1:2:0.75 (v/v) and homogenized in an ultrasonic (SB-103D) at a frequency of 100 KHz for 10 min. After centrifugation, the supernatant was stored in a vial. Cellular debris was re-extracted with the solvent mixture until the color was lost as described above, and filtered through syringe filters of 0.45 μm. The total extract was centrifuged at 10000 rpm for 5 min, the lower phase was collected in a pre-weighed vial and evaporated to be dried. The lipid content (%) and lipid productivity ($gL^{-1}d^{-1}$) of each sample were measured gravimetrically and calculated as follows [34]:

$$\text{Lipid content} = (WL \,/\, WA) \times 100$$

Where the lipid content (% dry weight), W_L is the weight of the total lipid extracted (g), and W_A is the weight of dry biomass (g).

$$\text{Lipid productivity } (\mathrm{g\,L^{-1}\,d^{-1}}) = \text{biomass productivity } (\mathrm{g\,L^{-1}\,d^{-1}}) \times \text{lipid content (\%)}$$

Carbohydrates were extracted using the Dubois method [35]. For this purpose, 2 mL water was added to 2 mg dry microalgae biomass. After centrifuge, 1 mL aliquot of the supernatant was added to 3 mL sulfuric acid

and 1 mL of 5% aqueous solution of phenol and incubated for 15 min at 95 °C. The calibration curve was obtained using D-glucose as a reference standard.

For the chlorophyll and carotenoid extraction, 50 mL methanol was added to 1.5 g microalgae and homogenized. After centrifuge, the supernatant was used for pigments quantification using the following equations [36]:

$$\text{Chlorophyll a } (Ca) = (16.72 \times A665.2) - (9.16 \times A652.4)$$

$$\text{Chlorophyll b } (Cb) = (34.09 \times A652.4) - (15.28 \times A665.2)$$

$$Total\ chlorophyll = Ca + Cb$$

$$\text{Total carotenoid} = \frac{(1000 \times A470) - (1.63 \times Ca) - (104.96 \times Cb)}{221}$$

Determination of fatty acid methyl esters (FAME) profile

Extracted lipid is converted into FAME by acid-catalyzed methylation, as previously proposed by Selvarajan et al. [19]. The FAME profile of microalgae was identified by a Gas Chromatography (PU4500-GC, Sweden) with a flame ionization detector and a CP Sil 5CB col-umn (30 m × 0.25 mm i.d., 0.25 µm film thickness) according to Selvarajan et al. [19]. The oven temperature was set from 160 °C to 230 °C at 8 °C min^{-1} and kept for 10 min. The carrier gas was He with a flow rate of 1.5 mL min^{-1}. Injector and FID temperatures were 230 °C and 205 °C, respectively. The fatty acid of species was identified by comparison with the retention time of standards and expressed as a percentage of the total fatty acids.

Prediction of biodiesel properties

Biodiesel properties investigated in this paper include KV, CP, SG, IV, CN, HHV, OS, LCSF, and CFPP, which were calculated based on FAME profile [5]. The KV, CP, SG, IV, CN, and HHV were estimated using the below equations:

$$KV = -0.6313\ X + 5.2065$$

$$CP = -13.356\ X + 19.994$$

$$SG = 0.0055\ X + 0.8726$$

$$IV = 74.373\ X + 12.71$$

$$CN = -6.6684\ X + 62.876$$

$$HHV = 1.7601\ X + 38.534$$

X is the average degree of un-saturation (ADU), which was calculated through the below equation:

$$ADU = \Sigma\ M \times Yi$$

M is the number of carbon-carbon double bonds and Yi is the mass fraction of each fatty acid, respectively. The OS was calculated in hours based on the below equation and Z is the content of linoleic and linolenic acids:

$$\text{OS} = \frac{117.9295}{Z} + 2.5905$$

The LCSF and CFPP are related to the saturation chain length in fatty acid, which is obtained according to the following equations:

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24)$$

193 $$CFPP = (3.1417 \times LCSF) - 16.477$$

194 **Statistical analysis**

195 The experiments were performed in a completely randomized design with three replications except for the fatty
196 acid components. Analysis of variance was done using the JMP 9 (John's Macintosh Project) software (U.S.A.)
197 and expressed as mean ± standard error (SE). The LS Means Student's (least-squares means differences
198 Student's test) was applied to assess the significant differences at $P < 0.05$. The correlation of data was
199 determined by Pearson's correlation coefficient (r). Principal Component Analysis (PCA) was performed using
200 PAST-3.14 [37] for multivariate analysis of fatty acid components in microalgae.

201 **Results and discussion**

202 **Morphological and molecular characterization of the isolated microalgae species**

203 In this research, the isolated microalgae were identified based on morphological characteristics and molecular
204 methods. 18SrDNA and *tuf*A regions were used to identify isolated microalgae. Many studies have emphasized
205 the necessity of multiple markers for the accurate identification of species in the Chlorophyceae [38, 39]. One of
206 the affective factors in species identification is the number of sequences deposited in GenBank. SSUrDNA
207 region is commonly used for microalgae identification [40]. The *tuf*A gene is a suitable marker for microalgae
208 identification. *Tuf*A region is a partial coding sequence and conserved region with a moderate evolutionary rate
209 [41, 42]. Vieira et al. indicated that the *tufA* gene was used as an appropriate choice for identification to the
210 species level in the genera *Scenedesmus* and *Desmodesmus* [43]. Ten microalgae specimens isolated were
211 tentatively named microalgae A, B, C, D, E, F, G, H, I, and J. The specimens showed the following
212 morphological characteristics: In the microalgae A and B, the cells were unicellular green microalgae, spherical,
213 cup-shaped chloroplast without flagella, 2 to 5 µm diameter in size, which were characteristics of *Chlorella spp*.
214 In the microalgae C and D, the cells were unicellular, usually spherical and sometimes elliptical, with two
215 flagella and a centralized nucleus, an eye spot on the front of the chloroplast, and two vacuoles on the front of
216 the cell, which were characteristics of *Chlamydomonas spp*. In microalgae E, the cells were spherical or oval
217 with two flagella of equal length, one eye spot usually at the end of the flagella, and a cup-like chloroplast,
218 which was characteristic of *Dunaliella spp*. In microalgae F, the cells were unicellular and spherical with a
219 nucleus, 8 to 10 µm diameter in size without flagella and pyrethroids, which were characteristics of *Picochlorum*
220 *spp*. Microalgae F had quite distinct features in terms of size from other *Picochlorum* species such as *P.*
221 *soloecismus* and *P. maculatum* [44, 45]. In the microalgae G, the cells were extremely green chloroplasts, a
222 pyrenoid inside the chloroplast, and a sheath wall, which were characteristics of *Tetraselmis spp*., but the
223 microalgae G differs in the size and pyrenoid position compared to those of the *Tetraselmis* species such as *T.*
224 *rubens* and *T. jejuensis* [46, 47]. In microalgae H, the cells were spherical or oval green cells with two flagella
225 and a central nucleus, sometimes without flagella and red, which were characteristics of *Haematococcus spp*. In
226 microalgae I, the cells was unicellular with a diameter of 3 µm, without flagella, spherical or cylindrical, and
227 containing yellow chloroplast, which were characteristics of *Nannochloropsis spp*. In microalgae J, the cells
228 were colonies from ovoid to spindle-shaped cells and needle-shaped and barbed cell poles, which were
229 characteristics of *Scenedesmus spp*.

230 Identification of microalgae species isolated from natural environments has the prospect of their application
231 in industrial purposes [13]. Because of the accuracy of species identification, the specimen was subjected to
232 18SrRNA and *tufA* sequencing. The length of the PCR products for *tuf*A and 18SrDNA, with the selected

primers, were 900 and 750 bp, respectively. The most similar species to the isolated microalgae with more than 94% query coverage and high similarity were obtained with a BLAST sequence match routine. According to BLAST results of 18SrRNA, ten isolated species (A to J) showed high similarity to *C. vulgaris* strain S3 (MK652782.1), *Chlorella* sp. Sp9 (MG237881.1), *C. raudensis* (DQ459878.1), *C. hedleyi* strain SLM0210 (OK030525), *D. salina* (MK774739.1), *Picochlorum* sp. RCC486 (KT861323.1), *Tetraselmis* sp. CCAP 66/75 (MG022704.1), *H. lacustris* (MH681993.1), *N. oceanic* (MN160639.1) and *Scenedesmus* sp. R6 (KF879583.1), in the NCBI database, respectively (Fig. S1, Supplementary Fig). For *tufA* gen, the BLAST results were similar and the specimens (A to J) matched with *C. vulgaris* isolate 962-1 (KR154281.1), *C. sorokiniana* isolate 275-1 (KR154269.1), *Chlamydomonas* sp. (MH590838.1), *Chlamydomonas* sp. (MH590838.1), *D. salina* strain CCAP 19/18 (GQ250046.1), *Picochlorum* sp. 'soloecismus' DOE 101 (MG552671.1), *Tetraselmis* sp. CCMP 881 (KU167097.1), *H. lacustris* (MG677935.1), *N. oceanic* (CP038136.1), and *S. rubescens* (HG514402.1), respectively (Fig. S1, Supplementary Fig).. Therefore, based on morphological characterization and the BLAST results, microalgae F and G were identified as new species with new names. Hence, they were named *P.bazangan* sp. nov., and *T. bazangan* sp. nov. According to Table 1, sequences obtained from *tuf*A and 18SrDNA regions were recorded in GenBank with the flowing accession numbers:

Growth parameters

Specific growth rate and biomass yield

Analysis of variance indicated that the specific growth rate (SGR) and biomass yield of all microalgae species exhibited remarkable differences. The specific growth rate and biomass yield ranged from 0.25 to 0.36 d^{-1} and 0.30 to 1.22 g L^{-1}, respectively (Table 2). Among all microalgae, the maximum level of specific growth rate was obtained in *C. vulgaris*. The specific growth rate in *P. bazangan* sp. nov. and *N. oceanica* was the minimum (lower than 0.3 d^{-1}). The highest biomass yield was obtained in *C. vulgaris* (1.22 g L^{-1}), followed by *C. sorokiniana* (1.03 g L^{-1}). Song et al. (2013) reported that the specific growth rate and biomass yield of *C. vulgaris* was 0.17 d^{-1} and 0.22 g L^{-1}, respectively [48]. Also, Santhakumaran showed that *Chlorella zachariaii* had a biomass productivity of 7.2 mg L^{-1} day^{-1}, which is comparable with the biomass productivity of the studied algae [13]. The specific growth rate of microalgae is considered an indicator of biological potential [49]. When the nutrients or light are not limiting factors and biomass productivity is low, the highest levels of SGR are observed [13]. Variation in the microalgae growth may be due to reflecting the genotypic differences among species [50]. Moreover, other factors such as temperature, light intensity, photoperiod, and the culture media composition have impacted the algae SGR [51].

Biochemical profiling

Protein, carbohydrate, lipid, and photosynthetic pigments

The protein, lipid, carbohydrate, chlorophyll, and carotenoid content showed significant differences at the stationary growth phase ($P < 0.05$) (Fig. 2). Similar to other research, protein, carbohydrate, and lipids were different in microalgae species [13]. Shin et al. and Safafar et al. indicated that the variation in biochemical components in microalgae depends on the species type, stage of growth, and medium culture [52, 53].

The protein content of microalgae ranged from 18.3% to 41.4% of dry weight. In this research, the microalgae *C. vulgaris* and *C. sorokiniana* showed the highest protein yield (41.4%, and 38.5%, respectively). Moreover, *Chlorella*, *S. rubescens* seem highly valuable as a cheap protein source. Tibbetts et al. (2015) showed that the protein content of *Chlorella sp.* with 53% of dry weight was more than other microalgae [54]. The

microalgae *Chlorella sp.* can be considered a food source for humans and many important aquaculture species [55]. In the study, there was a positive correlation between the growth rate and protein content ($r^2 = 0.82$; $P < 0.01$) significantly (Table 3). Raven and Giordano stated that this relationship is due to the effect of growth and RNA content on protein biosynthesis [56, 57].

As shown in Fig. 2, all species of studied microalgae were able markedly to accumulate lipids (more than 20% of DW). The highest lipid content was generated by *N. oceanica* and *C. hedleyi*, with 41.3% and 36.4% of DW, respectively. Chen et al. reported that *Nannochloropsis sp*. can accumulate more than 40% of lipid [58], whereas Guerra et al. showed that lipid content in *Nannochloropsis sp.* varied from 9.4% to 22.8% [59]. Moreover, culture conditions (such as stress situations) as an important factor can induce the production and accumulation of lipids in microalgae [60]. Correlation analysis in Table 3 showed a significant negative relationship between lipid accumulation and microalgae growth ($r^2 = -0.661$; $P < 0.01$), similar to results reported by researchers [61]. The growth rate will decrease when environmental factors stimulate lipid production [18]. Selvarajan et al. investigated that the best microalgae to biomass production did not correspond to higher lipid accumulation [19]. This negative relationship is probably due to lipid biosynthesis requiring a high metabolic cost. However, there are reports that show a positive relation between microalgae lipid accumulation and growth [62, 63]. In this research, *C. vulgaris* and *C. sorokiniana* had the maximum growth rate compared to other microalgae, although they accumulated the lowest lipid content with 20.3% and 22.1%, respectively. Mathimani et al., (2019) indicated that the lipid content of Chlorella species is within the range of 5% to 22%, similar to our results. Several microalgae such as *C. vulgaris* LC8 [23] and *Phaeodactylum tricornutum* [48] have a very high potential for utilization in the food industry and can be suitable candidates for biodiesel production [64].

The difference in total soluble carbohydrates among ten species was statistically significant ($df = 8$; $F = 4582.9$; $p < 0.05$) (Fig. 2). Carbohydrate content in the isolated microalgae ranged from 20% to 44.7% of dry weight. There was a negative relationship between carbohydrate accumulation, the growth rate, and other biochemical components (Table 3). According to the results, carbohydrate content in *C. raudensis* (44.7%) and *T. bazangan* sp. nov. (28.4%) was more than other isolated microalgae and can be considered a perfect source for bioethanol production by the microbial fermentation process [58]. The carbohydrate yield in some isolated microalgae was higher than what was reported by other researchers [65, 66]. Molino et al. observed that carbohydrate content in *H. lacustris*, *Chlorella sp.*, and *S. rubescens* were 6.30%, 5.30%, and 4.51% of dry weight, respectively [65]. These differences are probably dependent on the type of medium culture.

Chlorophyll and carotenoid content was variable in the isolated microalgae and ranged from 3% to 7% and 2% to 4% of dry biomass, respectively. According to the result, the highest chlorophyll and carotenoid content were obtained in *C. sorokiniana* and *D. salina*, respectively. Pigments have beneficial effects on human health due to antioxidant and anticancer activities [67]. The highest chlorophyll and carotenoid were observed in *Chlorella sp.* and *D. salina*, respectively. Molino et al. reported that the highest carotenoid content was obtained in *D. salina* (3.46%) [65], similar to our results. Pearson correlation in all microalgae showed that there was a positive correlation between biomass yield and pigments with $r^2 = 0.89$ for chlorophyll and $r^2 = 0.75$ for carotenoid ($P < 0.05$), significantly (Table 3). This relationship indicates that the pigments are an important indicator of photosynthesis and growth in microalgae. Chlorophyll directly absorbs and transforms sunlight into

energy, and carotenoids perform a photoprotection function [68]. The chlorophyll content of freshwater microalgae species generally differs from 1 to 5% of dry biomass [13].

Lipid productivity and fatty acid composition

Lipid productivity showed significant differences among the microalgae species ($P < 0.001$) (Table 2). The range of lipid productivity in studied microalgae was 7.45 to 11.64 g L^{-1} d^{-1}. The microalgae *N. oceanica*, *D. salina*, and *C. hedleyi* had lipid productivity of more than 10 g L^{-1} d^{-1}. Similar to the results, the different microalgae species generally have various abilities to accumulate lipids [69]. Lipid productivity was increased in the studied microalgae compared to other reports [18, 70]. Many research reported that oleaginous chlorophytes can accumulate high levels of lipid (average lipid content of 25% of dry weight) [48]. In large-scale cultivation, the most important parameters for algae selection as biofuel and biochemical are biomass yield and lipid productivity [71]. The highest biomass yield may not always mean the highest lipid productivity [13]. All the studied microalgae may be considered favorable industrial candidates because these species have high lipid productivity that is quite comparable with the already known high-efficiency species (0.60 to 9.46 mg L^{-1} day^{-1}) [51]. Optimizing the cultivation conditions of these algae is required to maximize the biomass and lipid yield, as potential algal species for biofuel production [72]. Much research confirmed that in addition to biomass productivity and lipid content, fatty acid profile are important quantifiable characteristics associated with biodiesel production [13, 31].

Based on Fig. 3, the FAME compositions in isolated microalgae were investigated, and they contained saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA), at the stationary growth phase. PUFAs contained the highest percentage of FAME, which flowed by SFAs, as reported by Molino et al. and Pratoomyot et al. [65, 73]. Myristic acid (C14:0), stearic acid (C16:0), and palmitic acid (C18:0) as SFA were detected in the studied microalgae. Palmitic acid was the main SFA in the microalgae lipids, which was in agreement with conde et al. [74]. The amount of palmitic acid among all algae was more than 20%, except *C. hedleyi* and *P. bazangan* sp. nov.. The *S. rubescens,* and *P. bazangan* sp. nov. microalgae had the maximum and minimum levels of palmitic acid with 29.4% and 13.2%, respectively (Table 4). The microalgae had MUFAs in the range 6.71% to 27.83%. MUFA consists of palmitoleic acid (C16:1), oleic acids (C18:1), and eicosenoic acid (C20:1). The highest and the lowest levels of palmitoleic acid were found in *N. oceanic* (16.2%) and *S. rubescens* (2%), respectively. Oleic acid existed in all of the studied microalgae and this fatty acid varied from 2% (*P. bazangan* sp. nov.) to 22% (*T. bazangan* sp. nov.) (Table 4). PUFAs contained from 28.75% to 69.18%. Portion of fatty acid in most microalgae was in order PUFA> SFA> MUFA (Table 4). The most important PUFAs are Linoleic acid (C18:2) and α-linolenic acid (C18:3) which existed in all microalgae. The precursor for the long-chain omega-3 PUFAs was α-linolenic acid [13]. *P. bazangan* sp. nov. and *C. sorokiniana* had the highest percentage of α-linolenic acid (27%), but the maximum level of linoleic acid was observed in *D. salina*.

All the studied microalgae contained notable and high levels of C16-C18 fatty acids (ranging from 60% to 85%) except *N. oceanic,* which had the lowest amount of about 40%. The highest amount of C16-C18 fatty acids was recorded in *H. lacustris,* followed by *T. bazangan* sp. nov. (82.3%) and *Chlorella sp.* (79%) (Table 4). Qualified biodiesel was enriched with the five most usual C16-C18 fatty acids, including C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid) [75]. The microalgae *S. rubescens, C. vulgaris, T. bazangan* sp. nov.*, D. salina,* and *P. bazangan* sp. nov. had the highest C16:0, C18:0,

C18:1, C18:2, and C18:3, respectively. Selvarajan et al. and Safafar et al. reported that *Chlorella sp.,* and *S. rubescens* are known producers of C16-C18 fatty acids [19, 53]. The suitable quantity of omega-3 and omega-6 fatty acids in most these microalgae species make them valuable biomass resources [13]. Although plant oils, fish oils, and animal fats are the major sources of the omega group of fatty acids in nature, According to the reports [76], microalgae can be considered an alternative source of valuable omega group of fatty acids. According to Kavale et al., (2017), the high ratio of omega-6 to omega-3 in food increases the risk of cancer, inflammation, and cardiovascular diseases [77]. In this research, the rate of omega-6/omega-3 observed in the biomass of all the species was in the range of 0.13 (*N. oceanica*) to 1.12 (*D. salina*), which fulfills the WHO standard (< 10) for utilizing the biomass as human food [63].

PCA-biplot was analyzed based on FAME composition to find out the fatty acid distribution among the isolated microalgae (Fig. 4). Seven fatty acids in four distinctly different groups were important variables to separate ten microalgae species. Selvarajan et al. reported that the three studied green microalgae, *Chlorella sp., Dictyosphaerium sp.*, and *Micractinium sp.,* were classified into two distinctly different groups (oleic acid and palmitic-linoleic acid) by PCA analysis [19]. The identified fatty acid groups were eicosapentaenoic acid (C20:5), palmitic acid (C16:0), stearic acid (C18:0), linolenic acid (C18:2 and C18:3), oleic acid (C18:1), and stearidonic acid (C18:4), while other fatty acids indicated little variations. According to Fig. 4, only *N. oceanica* had a high level of eicosapentaenoic acid, and this PUFA could distinguish *N. oceanica* from others. Shen et al. reported that eicosapentaenoic acid was characteristic fatty acid in *N. oceanic*, which was in agreement with this study [78]. Palmitic acid and stearic acid groups included *T. bazangan* sp. nov. and *C. vulgaris*. The linoleic acid group contained *C. sorokiniana*, *D. salina*, and *P. bazangan* sp. nov. The oleic acid and stearidonic acid groups included *C. raudensis* and *C. hedleyi*.

The basis on the fatty acid profile of the algal lipid, the biodiesel quality can be predicted [13]. The biodiesel properties depend on the hydrocarbon chain length of saturated and unsaturated fatty acids [18]. Saturated fatty acids and unsaturated fatty acids with a lower melting point than saturated fatty acids generally are considered for biodiesel biosynthesis [71]. Santhakumaran et al., (2018) indicated that the range of C14 to C18 fatty acids is the basic criterion of the biodiesel evaluation [13].

Physical/chemical properties of biodiesel

According to Table 5, ten main fuel properties, ADU, KV, SG, CP, CN, IV, HHV, OS, LCSF, and CFPP of the studied microalgae were determined. The ADU was calculated for all microalgae and ranged from 0.46 to 2.07. The highest and the lowest ADU were recorded in *C. raudensis* and *N. oceanica*. The KV and SG values in European and US biodiesel standards were 3.5 to 5 $mm^2 s^{-1}$ and 0.85 to 0.90 kg^{-1}, respectively. These fuel properties in all isolated microalgae were established in the standard ranges. The CP value ranged from -7.69 to 13.81 °C, and the highest and lowest levels were observed in *N. oceanica* and *C. raudensis*, respectively. According to the result, CP values in the microalgae with the low unsaturation degree, *C. vulgaris*, *T. bazangan* sp. nov., *N. oceanic,* and *S. rubescens,* were more than others. This study indicated that The CN value of all species ranged from 49.05 to 59.79, which can be consistent with the CN value of the American standard for biodiesel. In agreement with the European biodiesel standard, the CN values in *C. vulgaris*, *T. bazangan* sp. nov., *H. lacustris*, *N. oceanic*, and *S. rubescens* were more than fifty-one. According to the study, the highest CN was observed in *N. oceanic* (59.79) and *C. vulgaris* (55.38), whereas the lowest was obtained in *C. raudensis* (49.05) and *P. bazangan* sp. nov. (49.51). The present study, the IV for species *C. vulgaris* (96.30%), *T.*

bazangan sp. nov. (97.80%), *N. oceanica* (47.11%), and *S. rubescens* (117.21%) were lower than 120% coincided with the European biodiesel standards. The species with a high ADU, such as *D. salina* and *P. bazangan* sp. nov.*,* had the highest IV among microalgae. The HHV value in the studied microalgae was between 39 to 42 MJ/kg, which was in the range of the European biodiesel standard (less than 120 MJ/kg). The highest and the lowest HHV were observed in the microalgae *C. raudensis* and *N. oceanic,* respectively. The microalgae *C. raudensis*, *D. salina,* and *P. bazangan* sp. nov. had high ADU and HHV values. The OS was calculated in the studied microalgae and their value was within the Europe standard range (≥ 6 h) except *C. sorokiniana, D. salina, P. bazangan* sp. nov.*,* and *H. lacustris*. The highest and the lowest OS were observed in *N. oceanic* (56.43 h) and *P. bazangan* sp. nov. (4.93 h), respectively. The result showed that the OS level in *C. vulgaris, T. bazangan* sp. nov.*, N. oceanic,* and *S. rubescens* was more than in other studied microalgae. The LCSF and CFPP levels for all microalgae species varied from 2.07 to 9.95 and -9.97 to 14.78 °C. According to the study, the CFPP value in the microalgae *Chlamydomonas sp., T. bazangan* sp. nov.*,* and *S. rubescens* was in the European standard range (-5.00 to 13.00 °C). The maximum and minimum LCSF and CFPP were obtained in *C. vulgaris* and *P. bazangan* sp. nov. with 41% and 17% of SFA, respectively.

To predict the quality of biodiesel, in addition to the lipid fatty acid profile of algae, the characteristics of biodiesel properties like ADU, KV, SG, CP, CN, IV, HHV, OS, LCSF, and CFPP should be considered [13]. The quality of biodiesel mostly relies on the length of the carbon chain, position, and number of double bonds in the unsaturated fatty acids [79]. The long-chain saturated factor (LCSF) and cold filter plugging point (CFPP) depend on the concentration of saturated fatty acids that are indicative of diesel flow efficiency at low temperatures [80]. A comparison of the biodiesel properties of lipids from the three algal species revealed that the lipid from *Fasciculochloris boldii* alone was suitable for biodiesel preparation. The characteristic biodiesel quality features such as the highest ratio of saturated fatty acids and monounsaturated fatty acids to polyunsaturated fatty acids, the highest CN value (63.3), the highest score in LCSF (8.1%), and the lowest IV value (38.9) were observed from the fatty acid profile of *F. boldii*. The CN value as an index defines the quality of fuel ignition.

Therefore, the ADU of fatty acids had a strong relationship with biodiesel properties [81]. According to the results, CP, CN, KV, and OS values in the microalgae with the low ADU, *C. vulgaris*, *T. bazangan* sp. nov., *N. oceanic,* and *S. rubescens* were more than others. In agreement with our results, Hoekman et al. and Song et al. reported that the values of these parameters increase with elevation chain length and decline with increasing ADU [5, 48]. The IV, SG, and HHV decline with increasing and decreasing chain length and ADU, respectively [82]. In this study, the IV in species with low ADU was lower than 120%, coinciding with the European biodiesel standards. The *D. salina* and *P. bazangan* sp. nov. (with high ADU) had the highest IV (more than 120 g Iodine/100 g) and the lowest OS among microalgae. When IV in microalgae is more than 120 g Iodine/100 g, produced biofuel from them becomes more susceptible to oxidation [48]. Biodiesel production with high levels of unsaturated fatty acids, especially linoleic acid and α-linoleic acid, has a low OS, CN, and energy yield. This biofuel is not relatively stable at low temperatures and becomes solidified [81]. Based on biodiesel quality standards (the US and Europe), KV, SG, and HHV values in the studied microalgae were established in the standard ranges. The quality properties of good biodiesel consisted of high CN, OS, low KV, CP, IV, and CFPP. The high CN and low CP and KV are the main characteristics of fuel, which cause better combustion, less knocking occurrence, starting of the engine, and low nitrous oxide (N_2O) emissions [83]. High IV probably led

to enhanced polymerization of glycerides and lubricant deposition in engine [60]. Song et al. reported that PUFAs, with 3-6 double bonds, have poor oxidative stability and may not be used for biodiesel production [48]. A high level of palmitic, and stearic acid as saturated fatty acids in oil may cause crystallization at temperatures within the normal engine operation range and lead to poor LCSF and CFPP properties [84, 85]. Fevzi and Sehmus indicated that relatively low concentrations of long-chain SFAs and PUFAs in lipid composition lead to high-quality biodiesel, which had a good function at low temperatures [86]. The increase in the level of MUFAs leads to the best properties of ignition quality, combustion heat, OS, viscosity, CFPP, and lubricity [87]. Knothe reported that the important approaches to solving problems of biodiesel properties such as changing the fatty acids composition by genetic modification of raw material, physical method (stress conditions), or application of alternative raw material with various fatty acids profiles [88]. Also, CP (the parameters such as the cloud point) defined the biofuel quality. The values of CP of *C. vulgaris*, *T. bazangan* sp. nov., *H. lacustris,* and *S. rubescens* were within the limits of ASTM D6751. The CFPP values of *C. raudensis*, *C. hedleyi*, *T. bazangan* sp. nov.*,* and *S. rubescens* in the range of Europe (EN 14214), which indicated the biodiesel suitability at low temperatures.

Conclusion

Molecular analyses by 18SrDNA and *tufA* markers led to identified ten microalgae species from the saltwater lake (Bazangan, Iran). The microalgae *C. vulgaris* showed the maximum biomass yield, lipid productivity, and protein content, whereas *N. oceanica* accumulated the highest lipid content and percentage of omega-3 fatty acids. The biomass yield, lipid content, yield of omega-3 fatty acids, and C16-C18 fatty acids percentage in all the microalgae are comparable with industrially valuable species currently in use. The study of the biodiesel properties of algae lipids confirmed the biofuel potential importance of the isolated microalgae. Based on the important characteristics of biodiesel (high cetane number and low cloud point) and fatty acid profile, among the algae species isolated, *C. vulgaris*, *T. bazangan* sp. nov.*,* and *N. oceanic* can be considered suitable species with the potential ability to produce biodiesel. Therefore, the bio-prospecting of Bazangan Lake in the present study is to choose the best microalgae with biodiesel potential for industrial uses in the future.

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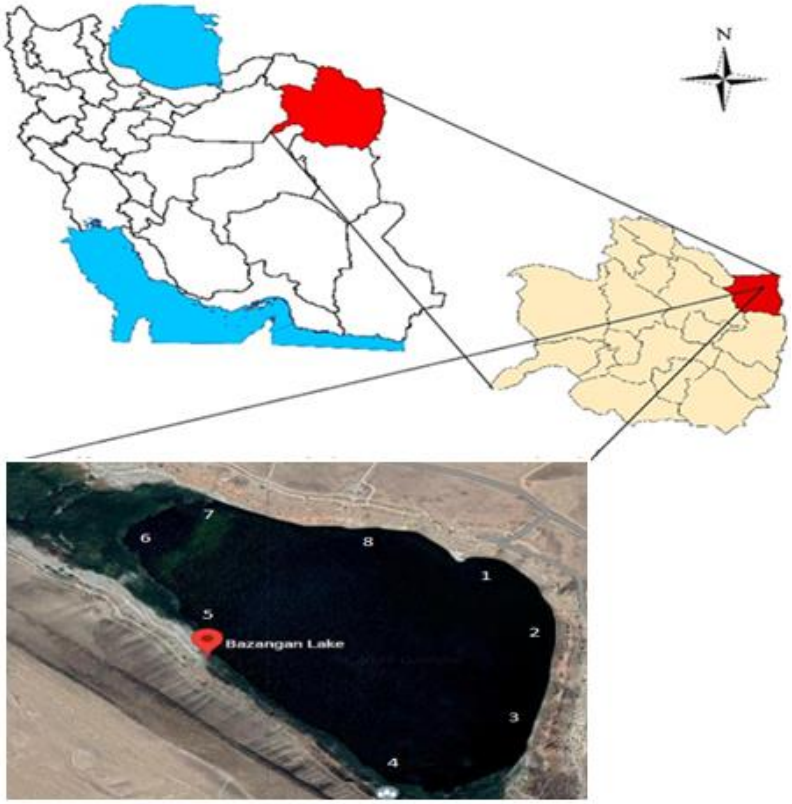
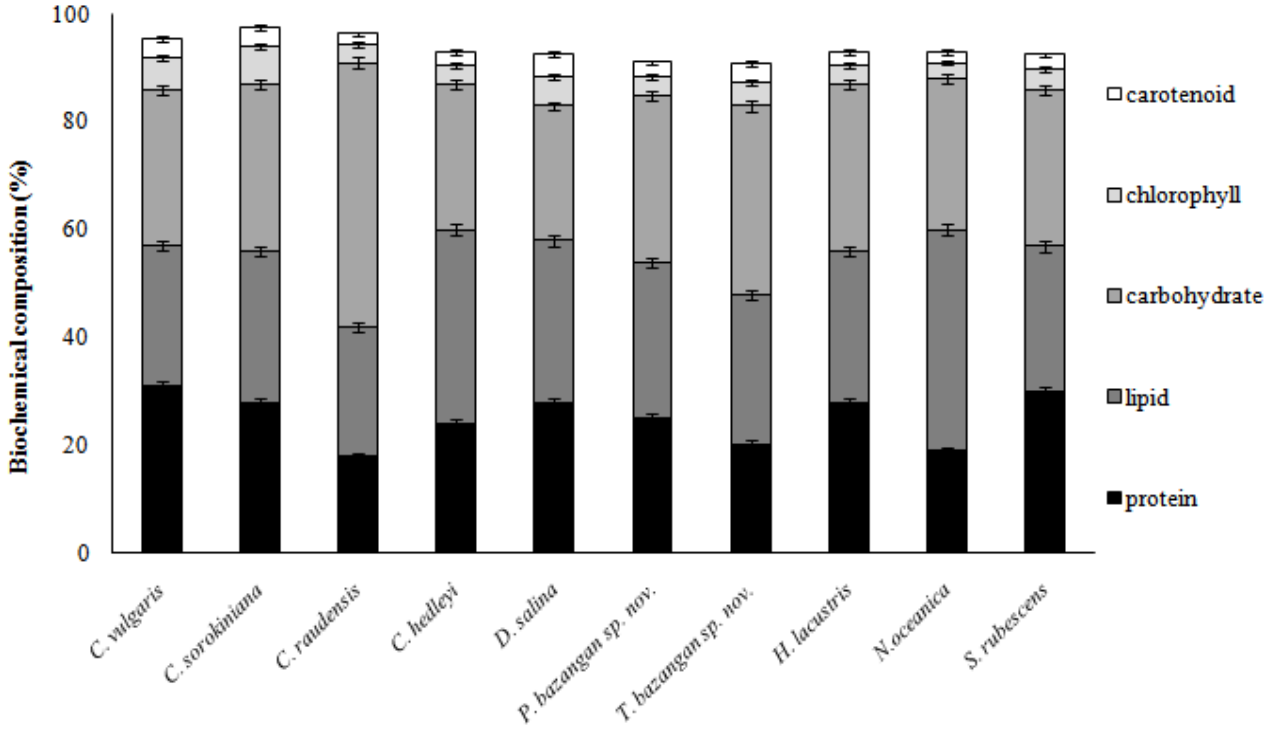


Fig. 1 Bazangan Lake location and the sampling sites



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724 **Fig. 2** The amount of protein, lipid, carbohydrate, chlorophyll, and carotenoid (% dwt ± SD (n = 3)) in isolated
725 microalgae. (Microalgae species, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlamydomonas*
726 *raudensis*, *Chlamydomonas hedleyi*, *Dunaliella salina*, *Picochlorum bazangan* sp. nov., *Tetraselmis bazangan* sp.
727 nov., *Haematococcus lacustris*, *Nannochloropsis oceanic*, and *Scenedesmu rubescens*).

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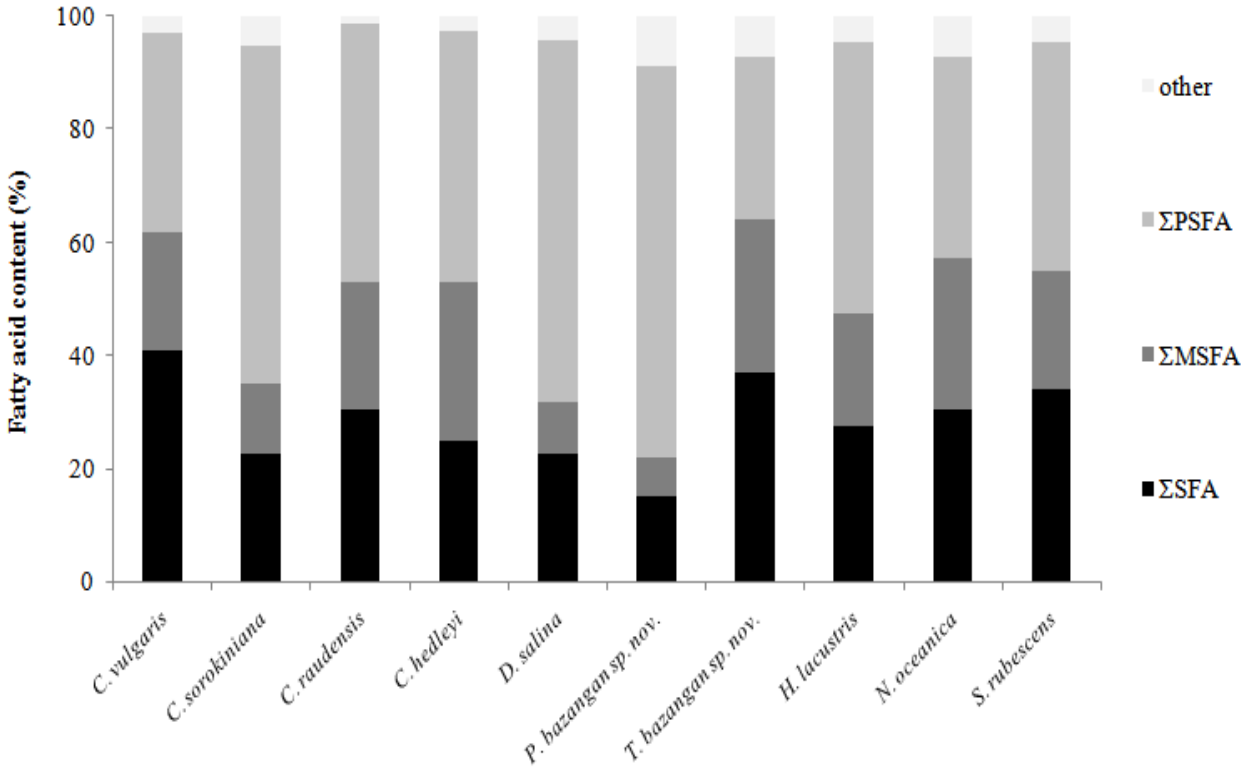
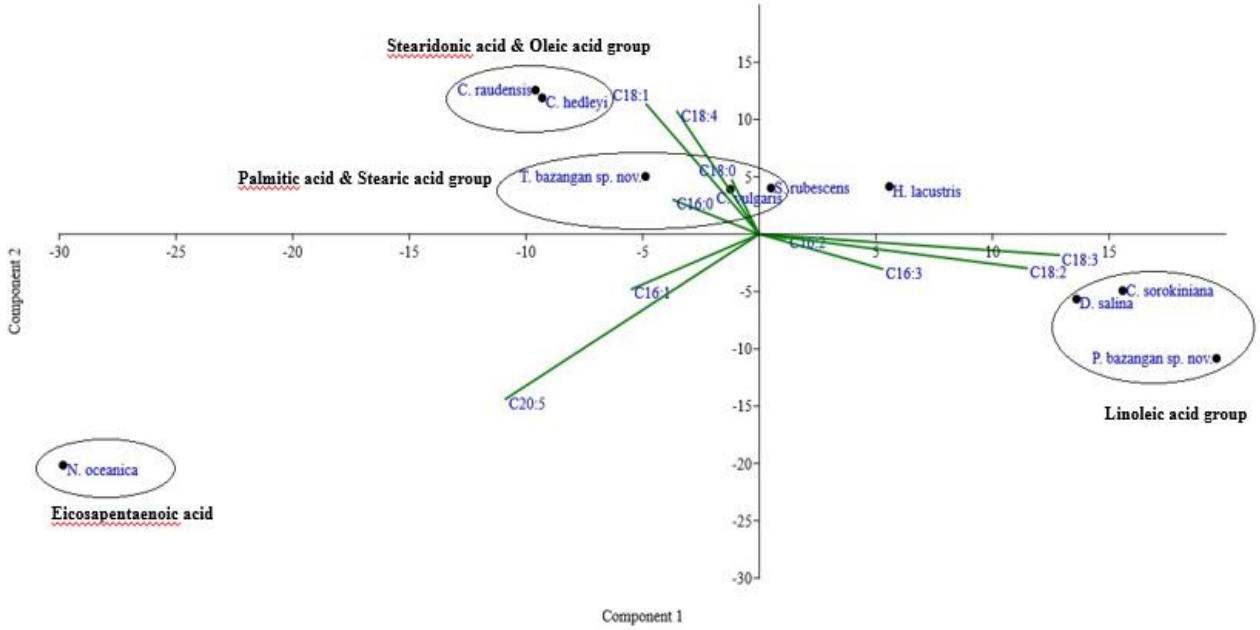


Fig. 3 The fatty acid compositions of the screened microalgae: SFA—Saturated fatty acids (C14:0, C16:0, C18:0); MUFA—Monounsaturated fatty acids (C16:1, C18:1, C20:1); PUFA—Polyunsaturated fatty acids (C16:2, C18:2, C20:2, C16:3, C18:3, C16:4, C18:4, C20:4, C20:5). (Microalgae species, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlamydomonas raudensis*, *Chlamydomonas hedleyi*, *Dunaliella salina*, *Picochlorum bazangan* sp. nov., *Tetraselmis bazangan* sp. nov., *Haematococcus lacustris*, *Nannochloropsis oceanic*, and *Scenedesmu rubescens*).



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761 **Fig. 4** Principal component analysis (PCA) bi-plots of 10 species of isolated microalgae for their FAME
762 composition. (Microalgae species, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlamydomonas*
763 *raudensis*, *Chlamydomonas hedleyi*, *Dunaliella salina*, *Picochlorum bazangan* sp. nov., *Tetraselmis bazangan* sp.
764 nov., *Haematococcus lacustris*, *Nannochloropsis oceanic*, and *Scenedesmu rubescens*)

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