Biodiversity of Cultivable Green Algae Collected from the Coast of Guilan

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Abstract

In this study, the cultivable algae in the coasts of Guilan province in south west part of Caspian Sea were isolated, purified and then morphologically and molecularly identified in February 2016. The separated samples were examined by optical microscopy and morphological analysis. Then, 18S rDNA gene primer pair was used for green algae polymerase chain reaction. Totally, 14 algae strains were identified. Among them, four strains were related to the algal branch of Chlorophyta. Considering the importance of the Caspian Sea as one of the largest aquatic habitats in the country, many studies have been conducted on the identification and study of phytoplankton and algae of the Caspian Sea. The seasonal variation of phytoplanktons in the southern basin of the Caspian Sea was studied. During a winter season, 31 species belonged to the Chlorophyta among 32 study stations. The results of this research show that the growth and diversity of the algae in the habitats is affected by seasonal fluctuations and changes in environmental factors such as temperature, salinity, food level, type of bed (due to spread range of sampling area) and a combination of them. In general, research has shown that some factors such as salinity, temperature, source of nitrogen and ambient oxygen, pH, heavy elements, UV rays and other environmental stressors affect the chemical composition and antioxidant activity in algae.

Keywords: Guilan Province, Algal isolation, Morphology, Molecular identification, Biodiversity.

Introduction

More than 70% of the Earth's surface has been covered with water. In all aquatic ecosystems, life is begun from producers, and animals are also dependent on these photosynthetic producers. In the aquatic reservoirs, the base of the nutrition pyramid is macrophyte and microphyte plants. Identification and study of the aquatic environments such as lakes, wetlands, rivers and other aquatic environments that are vital for the production of essential materials and the maintenance and proper utilization of them for environmental sustainability is a necessary issue (Ghahreman, 2004).

Microalgae are single-cell microorganisms that live in fresh or sea water and are present in a variety of shapes with a diameter or length of approximately 3 to 10 microme-

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ters. The term "microalgae" includes the prokaryotic and eukaryotic alga (Ferreira et al., 2013). Algae are important economically and environmentaly. Algae are at the base of the energy pyramid in the aquatic ecosystems and play a vital role as the main producers in the food chain, nitrogen stabilizers and providing suitable habitat for aquatic animals (Rabiei, 2007). By developing microalgal biotechnology in various fields due to capture various bio-active compounds have been enhanced. By increasing optimal microalgae cultivation knowledge, microalgae can become an economically sustainable and environmentally compatible resource for the production of optimal compounds, because there is the possibility of optimizing production in a controlled medium. Bioactive compounds derived from microalgae have anti-inflammatory, antimicrobial, antioxidant activity, and many more (de Morais et al., 2015). Various species with different ecological requirements show different compatibilities to the conditions of the origin of their life (Johnson et al., 2006). In this study, biodiversity of cultivable green algae in the coastal area of Guilan was investigated in the winter 2015.

Materials and Methods

Specifications of the location of study

The Caspian Sea is the largest brackishlake in the world, limited from north and northwest to Russia, from the west to Azerbaijan, from the east to Kazakhstan, from the southeast to Turkmenistan, and from the south to Iran, which contains about 40% of the world's lake waters (Kostyanoy and Kosarev, 2005). One of the most important features of the Caspian Sea is its biodiversity and physiochemical conditions, and because of the similarity of the ion composition of the Caspian Sea with seawater, many pure marine species can live in this sea (Aladin and Plotnikov, 2004). Sampling was carried out in winter 2015, from fourteen study stations in the southwest coast of the Caspian Sea in Guilan province. The geo-location of sampling stations described in Table 1. In the Figure 1, the sampling locations have been specified in the map with a check mark. For sampling at each station, two liters of water was collected in the plastic cap bottles. After 24-hour immobility of water, 20cc of its sediment was taken and used for morphological examination and cultivation. Samples of algae cells isolated from aqueous medium were observed under microscope. After identification of the genera, the microalgal name were transferred to the database of the microorganisms at the Iranian Biological Resources Center (IBRC).

Cultivation and isolation

BBM liquid culture medium, the dilution series up to 10-4 was prepared.

The tubes were held for 21 days in anoptic chamber with a temperature of 18°C and a light intensity of 2500 to 3000 lux with a cycle of 16 hours of brightness and eight hours of darkness (16:8).

Different dilutions were transferred to solid media and cultivated in a four-stage cultivation. Purification was performed using Richmond's various ecological and microbiological methods such as centrifugation, serial culture, antibiotic injections, sub-cultivating

Table 1. Location of sampling staions.

Row	stations	Longitude	latitude
1	Chaboksar	N36°97'09"99	E50°58'41"90
2	Near to Kalachai	N37°06'99"07	E50°41'69"52
3	Rodsar-Gaskar region	N37°12'32"88	E50°33'45"28
4	Chamkhaleh	N37°21'99"34	E50°27'13"43
5	Dastak	N37°39'37"10	E50°14'62"46
6	The sore of Caspian Sea	N37°47'92"78	E49°47'14"86
7	Bochagh	N37°47'04"12	E49°93'70"59
8	Sangachin	N37°52'22"27	E49°30'83"48
9	Gisoum	N37°67'76"39	E49°05'34"43
10	Talesh-Ghorough	N37°83'70"83	E48°97'58"92
11	Lisar	N37°96'22"65	E48°93'79"12
12	Havigh	N38°16'48"45	E48°90'63"54
13	Astara	N38°42'03"04	E48°88'04"57

Fig. 1. Distribution of studied stations have been shown by arrows.

with stereomicroscope, UV radiation, agar plate and serial culture (Richmond, 2008). Serial cultures were repeated six times, and then Imipenem antibiotic was used to purify the algae strains as the best antibiotic in order to obtain a single colony as well as to remove bacterial contamination from the culture medium (Andersen, 2005).

In the next step, morphological studies were performed using the examination of sample lamellas by Olympus optical microscope (CX 31). Photography was done by the same mi-

croscope equipped with a Canon Digital Camera 1680 PC. Morphological identification was performed using valid identification keys (John et al., 2002). Finally, molecular identification was carried out.

Molecular examination

Extraction of DNA and PCR

Genome isolation were performed using Sina gene's DNA extraction kit (for microorganisms). Molecular identification of the species was carried out using the 18S rDNA gene (Haddad et al., 2014). For molecular identification to replicate18S rDNA region, the primer pairs of SSU1 and 18S DR were used. Several primer pairs have been identified for the multiply sequence matching of the 18S rRNA gene known for PCR from eukaryotic microalgae. PCR replication of 25 ng DNA in a total volume of 25 µl using 0.8 µM dNTPs, 1.5 Mm MgCl2, 0.5 µM of each primer and 0.625 units of Taq DNA polymerase were performed under the following conditions (Berard et al., 200): PCR program includes initial separator temperature of 94°C for 3 minutes and 30 seconds, 36 cycles including a calibrator temperature of 94°C for 50 seconds, a connection temperature of 52°C for 50 seconds, and the development temperature of 72°C for 15 minutes. The *PCR product* was examined for 1% gel electrophoresis (Olmos et al., 2000). The final *PCR product* was sent to Pishgam Corporation and the sequence determination was carried out at Bioneer Corporation in South Korea.

Results

Desmodesmus communis

Cell shape is smooth, straight, or slightly curved multicellular colonies with cells in one or two rows and are usually 2, 4 and sometimes 8 cells and rarely 16 cells, sticking together from their sides and aligning each other in parallel (linear arrangement) or they have intermittent arrangement, and stick together from the end. The cells are elongated or cylindrical, ovoid, elliptic to ovoid, with round and short heads, or with head-like zones that are narrowed towards heads with an open angle and always have tall appendages or jagged edges.

Cells are smooth, straight, or slightly curved multicellular colonies, which the cells are arranged in one or two (and rarely in three) rows. They are usually 2, 4 or 8 cells, and in very few cases 16 or 32 cells, which are joined together in their sides and are aligned in parallel (linear arrangement) or they have intermittent arrangement, and stick together from the head. They are usually surrounded by mucilage membranes.

The cells are elongated or cylindrical, ovoid, elliptical to ovoid, and usually with rounded heads.

They have smooth and polished walls with granularity. Cells are single, rarely seen in groups, in the form of spherical, elliptic, and usually seen with thin, smooth walls. Chloroplasts are wall-shaped, often single, cupped, striped or tube and rarely with narrow or lattice sections, usually with one pyrenoid, and sometimes with a starchy coating.

Lemmermannia tetrapedia

The planktonic green algae, four cells stick together from center in a square arrangement.

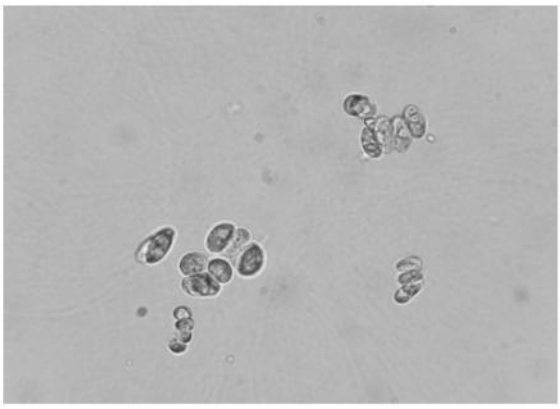

Fig. 2. *Desmodesmus communis* under light microscopy (1000 magnification) *Tetradesmus acuminatus.*

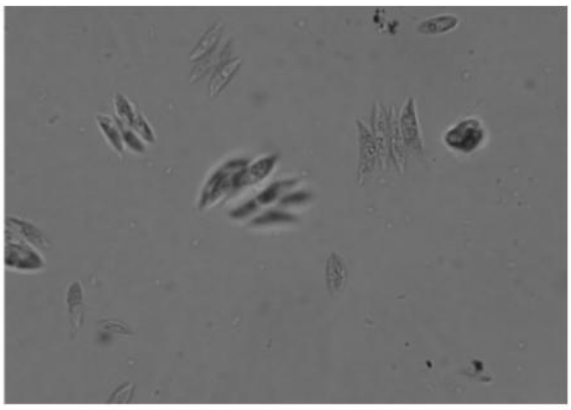

Fig. 3. *Tetradesmus acuminatus* cells under light microscope (1000 magnification) *Chlorella emersonii.*

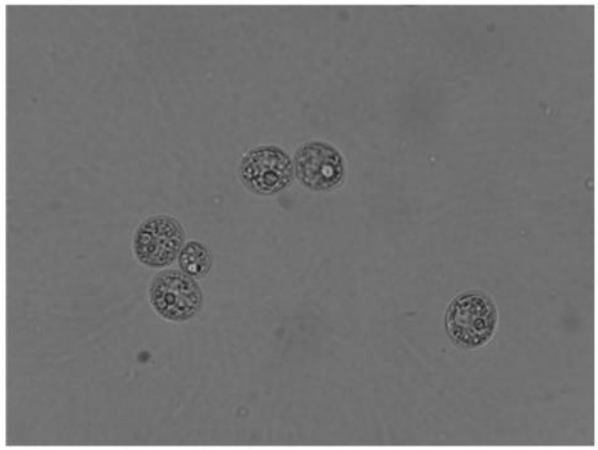

Fig. 4. *Chlorella emersonii* under light microscopy (1000 magnification).

Sometimes incoenobia, the number of compound cells reaches 16. The cells are tightly clamped, and are tangent to each other in the central area or have a small hole.

The cells are triangular to ovoid. Chloroplasts are single with walls with or without pyrenoid. Non-sexual proliferation is performed by autosporulation (four autospores per sporangium) and their sexual reproduction is unknown. This genus is distinguished by the nucleotide sequences of the18S rRNA gene (Bock et al., 2013).

PCR product

18S rRNA region PCR results produced bands with a molecular weight of 1750 nucleotide pairs for isolated samples using the primer pairs of SSU1 and DR 18S related to the 18S rRNA gene. In this study the 18S rRNA sequencing analysis results for algae samples showed different bands in *PCR product* which indicated the existence of different species.

Discussion

Considering the importance of the Caspian Sea as one of the largest aquatic habitats of the country, some researches have been done

on investigation and identification of Phytoplankton and macroalga in Caspian Sea. Due to the complex nutrient requirement of algae, it is not possible to grow all the species in the same culture media. In a study on the phytoplankton composition in the southern basin of the Caspian Sea with 6 study stations, totally 33 species belonged to the chlorophyta branch were identified (Tahami, 2012).

Among the obtained cultivatable strains, 17 species were from the chlorophyta branch. The lowest and highest density of phytoplanktons was reported in spring and winter respectively (Mahmoudi et al., 2017). Also, a study was conducted (Afraei et al., 2016) on ecological relationships between phytoplankton and zooplankton in which 19 species of chlorophyta were identified. In this study, 16 percent of all the obtained strains belonged to chlorophyta (Afraei et al., 2016). In a comprehensive study carried out in several periods, the total number of strains obtained during the years 1996, 2008, 2009, and 2010 was 80, 195, 191 and 181 strains, respectively, of which 38, 30, 6 and 31 species were chlorophytes, respectively (Nasrollahzadeh et al., 2014). Considering that sampling was performed in the winter, and compared with previous studies, it was expected that *Bacillariophyta* species were dominant and included *Cyclotella* sp., *Nitschia microesphala, Mayamia permitis, Nitschia palea, Nitschia microesphala, Tryblionella* sp., and Navicula salinarum of 14 strains identified, only four species belonged to chlorophyta.

Breeding of microalgae requires control of all growth factors such as nutrients, pH, temperature, oxygen concentration and carbon dioxide and intensity of light. The presence of sufficient nutrients in the microalgae culture medium differ according to type of algae and its species in fresh and salty water. Some algae grow in a special culture medium and some in a public culture medium (Ganjian et al., 2013). Heidari et al. (2011) investigated different effects of nitrate and ammonium on *Scenedesmus quadricauda* from chlorophyta species in BBM medium. The breeding of this species was carried out in seven different treatments at 22°C, which the results showed that this species has the capability to withstand 75mM of nitrate, and the highest algae biomass was obtained in 15mM of nitrate. Therefore, this concentration can be used to improve the chemical composition of the BBM medium (Heidari et al., 2011). In another study conducted to investigate the rate of chlorophyll a, b and total carotenoid isolated from Golestan coasts, the cultivation of algae strains was also done

Table 2. Biological classification of strains identified for chlorophyta in the present study

Microalgae name	Class	Order	Family
Desmodesmus communis	Chlorophyceae	Sphaeropleales	Scenedesmaceae
Tetradesmus acuminatus	Chlorophyceae	Sphaeropleales	Scenedesmaceae
Chlorella emersonii	Trebouxiophyceae	Chlorellales	Chlorellaceae
Lemmermannia tetrapedia	Trebouxiophyceae	Trebouxiophyceae	Trebouxiophyce

Table 3. Results of molecular identification of algae species

Access number of genetic database	Similar strain	Similarity rate
JQ356706.1	*Lemmermannia tetrapedia*	96%
KP726233.1	*Desmodesmus communis*	99%
LC192133.1	*Tetradesmus acuminatus*	99%
FR865661.1	*Chlorella emersonii*	99%

Table 4. Results of molecular identification of algae species

Access number of genetic database	Similar strain	Similarity rate
JQ356706.1	*Lemmermannia tetrapedia*	96%
KP726233.1	*Desmodesmus communis*	99%
LC192133.1	*Tetradesmus acuminatus*	99%
FR865661.1	*Chlorella emersonii*	99%

in the BBM medium with biomass method, in this way, the collected specimens were transferred to the laboratory and deposited after 24 hours. In that study, four strains of green algae, including *chlorella vulgaris, chlorella surkinia, chlamydomonas diariana* and *selenstrum* were identified (Hassan Soltan et al., 2014).

A study was conductedto optimize the medium of the *Chlorella vulgaris* microalgae in order to increase starch production in Iran in 2014. In that study, 5 mg of *Chlorella vulgaris, Scenedesmus dimorphus* and *Scenedesmus quadricauda* were cultured in test tubes containing BBM and Z8, and optical absorption and biomass of microalgae were investigated. The results showed that there was a significant difference between BBM and Z8 culture media ($p=0.034$), and therefore, BBM medium was selected as the most suitable medium for *Cholrella vulgaris* culture. The slope of the line obtained for BBM medium was 0.545×10^6 cells/Ml and for Z8 medium was 0.454×10^6 cells/Ml. The results of the study did not show a significant difference in optical absorption in the above media (Ramezani et al., 2016).

In a study conducted in India to optimize various growth media to freshwater microalgae for biomass production it was found that the growth of the species belonged to Scendesmus was significant in the BBM medium, while the growth of *Chlorella* species in the Chu medium was in optimal mode. All strains studied in this study had optimal growth in the acid BBM and BG-11 medium, but in Hoagland's modified medium, microalgae did not have significant growth (Ilavarasi et al., 2011).

In the present study, the BBM medium was used for culture and purification of microalgae. The type of culture medium can affect biomass, growth, cell shape and pigments.

The main problem with the cultivation and preparation of algal biomass is the presence of a large number of organisms and the contaminations that need to be eliminated in their own processes. According to the results, imipenem antibiotic inhibits bacteria that are recommended for purification of algae culture (Noroozi et al., 2016).

Considering the 96% similarity of the *Lemmermannia tetrapedia* species in the NCBI, it seems that this is a new species. To ensure this, additional tests should be performed, including sequencing of rbcL and ITS genes, as well as physiological tests and SEM and TEM imaging. In the present study, 18S rRNA sequences were used for molecular identification. In order to confirm the results further, the evaluation of other genes, such as the nuclear-adjacent ITS gene and the chloroplast subunit of rbcL gene are also suggested. In addition, a complete colony of 18S rRNA area is recommended for complementary studies.

In general, research has shown that in algae, factors such as salinity, temperature, source of the environmental nitrogen and oxygen, PH, heavy elements, UV radiation and other environmental stresses affect the chemical composition and antioxidant activity. Thus, the individual's differentiation of a species collected at different times and locations depends on different environmental factors and changes in the physical and chemical parameters of water. In present study, sampling was done only in winter, we need further research to collect more morphological and molecular data in other seasons to explain ecological conditions and flora of this area.

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