Morphological and Molecular Identification and Antioxidant Measurement in Cultivated Algae from Unusual Waters of Semnan and Garmsar (Semnan, Iran)

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Abstract

The debate on biodiversity is one of the most important issues in today's world. In this study, we focused on the examination and characterization of cultivable algae in the unconventional waters of Semnan and Garmsar. Sampling was conducted at eight different locations and employed microscopic and molecular techniques to identify the algae's morphological and genetic features. Through microscopic analysis, we were able to identify the visible and structural traits of the algae accurately. The molecular identification involved analyzing the 18S rRNA and 16S rRNA genes and conducting BLAST searches on the NCBI database. Additionally, we constructed a phylogenetic tree using MEGA software (version 6). Out of the 10 strains identified, six belonged to the chlorophytes, while four were classified as cyanophytes. The antioxidant activity of the isolated strains was also evaluated using the ferric-reducing antioxidant power (FRAP) method. Furthermore, the total antioxidant activity of some strains was measured after inducing changes in cell color. The morphological and molecular methods helped us identify various genera, including *Leptolyngbya* sp., *Scenedesmus* sp., *Coelastrella* sp., *Dunaliella* sp., and *Pseudanabaena* sp. The results indicated a higher prevalence of Chlorophyta compared to other groups. Since there is a lack of previous research on algae identification in the Semnan Province, this study provides valuable insights into the local genetic resources of algae, which can serve as a foundation for future investigations.

Keywords: Biodiversity, FRAP assay, Isolation, Photosynthetic Organisms, Purification

Introduction

There are several floristic and systematic studies focusing on the flora and systematics plants in Iran including recent works by Rasouli–Dogaheh et al. (2023), Naseri et al. (2022), Naseri et al. (2020). Algae, in particular, play a crucial role in both aquatic and terrestrial ecosystems by providing oxygen, food, energy, and essential

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biomolecules. The taxonomic diversity of algae within a biological community is a significant indicator of its functional, evolutionary, and ecological processes, as well as the overall stability of the ecosystems. Conducting floristic studies on algae in various ecosystems helps unveil the taxonomic diversity and species composition of these communities. Moreover, algae have practical applications, such as their use as biosensors or for bioremediation purposes. Algae exhibit heightened sensitivity to environmental changes, including physical, chemical, and biological alterations. This sensitivity often allows for the detection of algal responses before those of higher organisms, making them valuable indicators of environmental changes (Atazadeh et al., 2021).

There is no simple functional definition for biodiversity however, it generally refers to the variety of life forms present on Earth. It encompasses the diversity of species, ecosystems, genetics, and habitats among living organisms.

Microalgae constitute a group of uni- and multi-cellular microorganisms, which use light and inorganic substances, including carbon dioxide, nitrogen and phosphorus, to produce biomass and compounds that are useful for their survival (Schuelter et al., 2019). These microalgae can grow in diverse conditions, including high or low temperatures, extreme pH levels, and the presence of metallic ions, high radiation, and coexistence with other microorganisms in their habitats. Despite their significant potential for biotechnological applications, the potential of microalgae has received little attention thus far. The conditions in which microalgae develop in extreme natural environments are often deviate from optimal growth conditions (Varshney et al., 2015). Such conditions include variations in salinity, pH levels, pollutants, light availability, and the concentration of dissolved metals (Amils et al., 2014).

Cyanobacteria and algae have diverse applications in various fields, particularly in agriculture and industry. They serve as a valuable food source, fertilizer, biological control agents, and active biological additives like ω-3 fatty acids (Siahbalaei et al., 2020). They are also utilized in biofuel production and sewage treatment. Additionally, these microorganisms produce a wide range of bioactive compounds, known as secondary metabolites, through various biological activities. These compounds possess important properties in medicine, industry, and agriculture, including antiviral, antimicrobial, antifungal, anti-malarial, anti-tumor, anti-inflammatory, and diabetic properties (Gupta et al., 2013; Siahbalaei et al., 2021).

Microalgae are presently being used commercially as a source of carotenoid antioxidants for food additives, dietary supplements, and cosmetic applications. (Pulz and Gross, 2004; Khalili et al., 2019; Khalili et al., 2020). These antioxidants play a crucial role in protecting microalgae against reactive oxygen species (ROS) and effectively delaying oxidation. ROS

is generated during biological reactions and electron transfer chains in chloroplasts and mitochondria, as well as due to environmental stresses like UV radiation, high light intensity, temperature fluctuations, salinity changes, exposure to heavy metals, and pH variations (Coulombier et al., 2021; Ugya et al., 2020). ROS generally refers to oxygen derivatives that contain at least one unpaired electron, making them highly reactive compared to oxygen itself (Demidchik V., 2015).

Microalgae possess various antioxidant molecules, including ascorbic acid (vitamin C), glutathione, tocopherols, phenolic compounds, carotenoids, and a range of antioxidants such as MMA (mycosporins-like amino acids).In the field of algae identification, modern molecular techniques serve as valuable tools to complement microscopic identification. These techniques offer explicit and unambiguous identification based on evolutionary markers (Eland et al., 2012). The 16S rRNA and 18S rRNA genes are commonly employed as genetic markers for the classification of cyanobacteria and eukaryotic algae, respectively. These genetic markers aid in the investigation of biodiversity among cultivable algae inhabiting unconventional environmental conditions. Additionally, they facilitate the evaluation of morphological and molecular identification techniques for this group, as well as their potential for antioxidant production.

Overall, this study aims to explore the biodiversity of cultivable algae thriving in non-traditional environmental conditions. It also seeks to assess the effectiveness of both morphological and molecular identification methods for this group, while investigating their capacity for producing antioxidants.

Materials and methods

Sampling

Sampling was performed on 6 September 2016 at 4 stations with unusual water sources (water with high salinity, such as high sulfur water, hot tap water, and water with an acidic or basic pH). The sampling sites are in the towns of Semnan and Garmsar.

Isolation and purification of strains

The serial dilution method was used to dilute the samples and they were cultured in BBM, BG, and MOH medium. The inoculated plates were placed at 23°C, 2700 lux light, and 12-12 h illumination time. Various antibiotics such as imipenem (110 micrograms per ml), nystatin (100 µg/1 ml), and cycloheximide (100 µg per 1 ml) have been used in the medium to inhibit the growth of undesirable organisms. (Richmond, 2023). Purified algae obtained from single colonies identical in morphology, shape, and apparent color were examined under optical and stereomicroscopy.

Morphological study of purified strains

An optical microscope (Nikon Eclipse 80i) was used to observe morphological characters such as cell shape, cell length and width, presence or absence of envelopes and pigments, and molecular strain identification.

Molecular identification of strains

The universal primer of 16S rRNA and

18S rRNA along with the gene polymerase chain reaction for cyanobacteria and micro-algae respectively were used for molecular identification of strains.

GeneAll Exgene™ Plant SV mini (117-101,117-152) kit was used to extract the genome of three strains including Tlsj7, Bnk9, and Hbr13, and DNA extraction of other strains was done according to the Liu et al (2000) method.

According to the protocol, master mix buffer (Taq 2x master mix Red 1.5 mμ $MgCl_2$) was used to amplify 16S rRNA and 18S rRNA genes; buffer volume is 25 µl, primer volume is 1 µl and eukaryotic DNA volume is 4 µl (if we have prokaryotic DNA the volume is 1 µl) compared to using l water two mark, the final volume was 50 µl.

To test the PCR results, the reaction product was run on the electrophorese on agarose gel 1% close to the 10 Kbp ladder. The length of the 16S rRNA gene was around 1000 bp and the length of the 18S rRNA gene was roughly 1400 bp. At that point, the bands were observed in the gel duct machine using UV light. Finally, the PCR product was sequenced in South Korea through Pishgam Biotech Company. The sequencing results were investigated and modified using the Bioedit program and then were compared with available sequences of environmental samples using Blast search on the NCBI database. The levels and the phylogenetic tree were obtained using ClustalW and the MEGA program (version 6), respectively. (Etemadi-Khah et al., 2017).

Total antioxidants activity of strains by FRAP method

Ferric Reducing Antioxidant Potential (FRAP assay) was first described by Benzie and Strain (1976). The ferric-reducing power of algae extracts was determined using the FRAP assay. The FRAP reagent consisted of 10 volumes of 300 mM acetate buffer, 1 volume of 20 mM $FeCl_3$, and 1 volume of 10mM 2,4,6- tri (2-pyridyl)-s-triazine (TPTZ) solution. TPTZ and $FeCl_3$ solution are unstable and toned to protect against light. To prepare the FRAP reagent, 5 ml of fresh TPTZ solution was added to 5 ml of $FeCl_3$ solution and 50 ml of acetate buffer solution respectively, to give a yellow-brown solution. This solution is unstable and unusable after 30 minutes. The solution was covered with foil because it is sensitive to light.

Two hundred fifty µl of diluted methanolic extract from lyophilization of algal cell sediment was added to 1.5 ml of FRAP reagent. The samples were kept in the dark for 20 m. After incubating for 10min at 37 °C in a water bath, the absorbance was measured at 593 nm. All measurements were replicated three times (Miranda-Delgado et al., 2018) (Hajimahmoodi et al., 2010).

Three isolates Habg3, Tlsj7, and Abg2 were evaluated again for antioxidant activity after changing the color of the cell culture from green to orange and brown.

Preparation of standard solution of iron sulfate

0.278 g of $FeSo_4.7H_2O$ was dissolved in 100 ml of distilled water and the absorbance of its different concentrations of 125, 250, 500,

750 and 1000 µl were read at 593 nm. From the scheme, an absorption diagram and a linear equation of different concentrations drawn using an Excel program were obtained. Once the line equation is obtained, the average of three absorbance replicates for each strain is used in the ferrous sulfate standard curve equation. Thus, a reduced amount of iron with total antioxidant content was obtained in the algal extract of the strains (Thaipong et al., 2006).

Results

The geographic location and physicochemical properties of each station are specified in Table 1 and Figure 1Semnan Ab-Garm Fountain pool, the pool outside Ab-Garm Fountain, Ab-Garm River, Talkhab-e Lasjerd, Bon Kooh, Iwanaki, and Hableh Roud were the main sampling stations. Details of strains with their regional codes are depicted in Table 2. Ten species from investigated locations were identified which are displayed in Figure 2.

Table 3 lists the primers used in this study. These primers were specifically designed for the polymerase chain reaction (PCR) amplification of the targeted genes. Furthermore, Table 4 displays the temperature program utilized during the PCR process for amplifying these two genes. Figure 3 demonstrates the results of

Table 1. Geographic location and physicochemical properties of stations

Name of stations	x & y	T °C	debi (m^3/s)	pH	*EC (µS/c)	Hco_3 (mg/l)	Cl (mg/l)	So_4 (mg/l)	Ca (mg/l)	Mg (mg/l)	TDS (mg/l)	Na (mg/l)
Ab-garm Fountain	35-657597 53-189693	37	9.5	7.9	12800	4.05	121	35.23	22.4	12.6	9460	124.3
Talkhab-e Lasjerd	35-399886 53-083287	32	-	7.12	12000	-	90	30	18	4	8010	84
Hableh Roud	35-302510 52-425531	30	5.5	7.32	5210	3.05	23.8	28.18	14.2	12.27	3490	25.52
Bon Kooh	35-18-00 52-26-00		2.98	8	2400	3.1	125	10	5.2	4.6	2014	100

*µSiemens/cm

Fig.1. The map of sampling stations

Table 2. Name and codes of the region

Codes	Name of region
Abg1	Ab-garm pool1
Abg2	Ab-garm pool2
Hzabg3	Ab-garm pool outside3
Rabg5	Ab-garm River5
Tlsj7	Talkhab-e Lasjerd7
Tlsj8	Talkhab-e Lasjerd8
Bnk9	Bon Kooh9
Eyv10	Iwanaki10
Hbr13	Hableh Roud13
Hbr14	Hableh Roud14

electrophoresis, where bands were observed in the region of 1800bp for the 18S rRNA gene and 1000bp for the 16S rRNA gene. The presence of these bands confirms the successful amplification of the target genes through PCR, indicating the accuracy of the PCR process.

Figures 4 and 5 present the outcomes of sequence blasting for molecular identification, along with the subsequent construction of phylogenetic trees. Figure 4 specifically displays the results obtained from blasting the sequences of the 18S rRNA gene, while Figure 5 represents the outcomes of blasting the sequences of the 16S rRNA gene. The phylogenetic trees plotted based on these results provide insights into the evolutionary relationships and relatedness of the studied microalgae species.

Using both morphological and molecular identification methods, the 10 strains were successfully identified, as indicated in Table 5. Among these strains, four were classified as belonging to the cyanophyta, specifically *Leptolyngbya* sp., *Pseudanabaena* sp., and *Leptolyngbya tenuis* (Gomont) Anagnostidis & Komárek (1988). The remaining six strains were identified as members of the Chlorophyta, including *Scenedesmus vacuolatus* (Shihira et Krauss) Kessler et al., 1999, *Scenedesmus* sp., *Scenedesmus obliquus* Nägeli 1849, and *Dunaliella* sp.

Strain Tlsj8, among the identified samples, displayed lower morphological and genetic similarity with its closest genus, *Leptolyngbya* sp., in terms of percentage of similarity and overlap. This suggests that strain Tlsj8 could potentially be a candidate for a new taxon. Further studies are recommended to investigate and characterize this strain in more detail.

Table 6 provides information on the absorption of desired concentrations. Additionally, Figure 6 illustrates the standard curve obtained from a 10 mM Ferrous Sulfate solution, along with the corresponding line equation. These measurements and

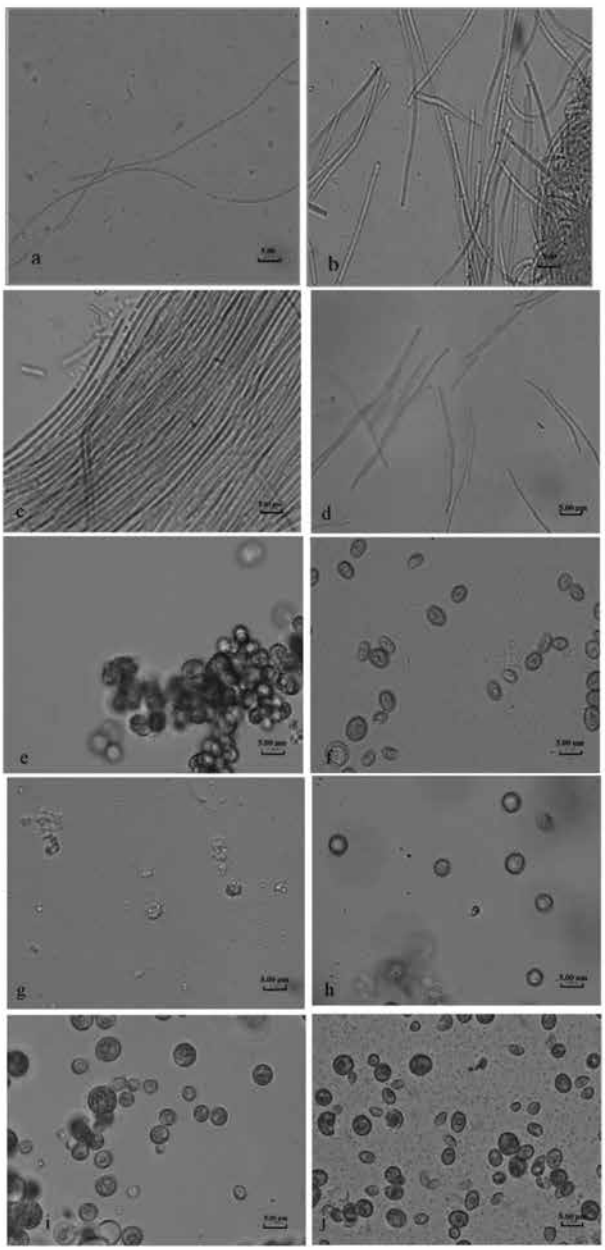

Fig. 2. the microscopic images of 10 strains (scale bar = 10μm), a. *Pseudanabaena* sp. (Abg1), b. *Leptolyngbya* sp. (Abg2), c. *Leptolyngbya tenuis*. (Rabg5), d. *Leptolyngbya* sp. (Tlsj8), e. *Scenedesmus vacuolatus* (Habg3), f. *Scenedesmus obliquus*(Tlsj7), g. *Dunaliella* sp. (Bnk9), h. *Dunaliella* sp. (Eyv10), i. *Scenedesmus obliquus* (Hbr13), j. *Scenedesmus* sp. (Hbr14).

Table 3. Name and sequence of primers (Alverson & Kolnick, 2005; Etemadi-Khah et al., 2017)

Sequence	Name of primer	gene
5′ GGGGAATYTTCCGCAATGGG 3′	CYA359F	16srRNA
5′ACGGGCGGTGTGTAC 3′	PCR Riverse	
5′ AACCTGGTTGATCCTGCCAGT 3′	SSU1-478	18srRNA
5′ CCTTGTTACGACTTGACCTTCC 3′	ITS1DR	

Table 4. PCR cycle for 18srRNA and 16srRNA genes (Alverson & Kolnick, 2005)

Steps	Temperature °C		Time		cycles	
	16srRNA	18srRNA	16srRNA	18srRNA	16srRNA	18srRNA
Initial Denaturation	95	94	7 min	3.5 min	1	1
Denaturation	95	94	40s	50s	33	35
Annealing	52	55	1min	50s	33	35
Extension	72	72	1:30min	80s	33	35
Final extension	72	72	10min	30min	1	1

Figure 3 demonstrates the results of electrophoresis, where bands were observed in the region of 1800bp for the 18S rRNA gene and 1000bp for the 16S rRNA gene. The

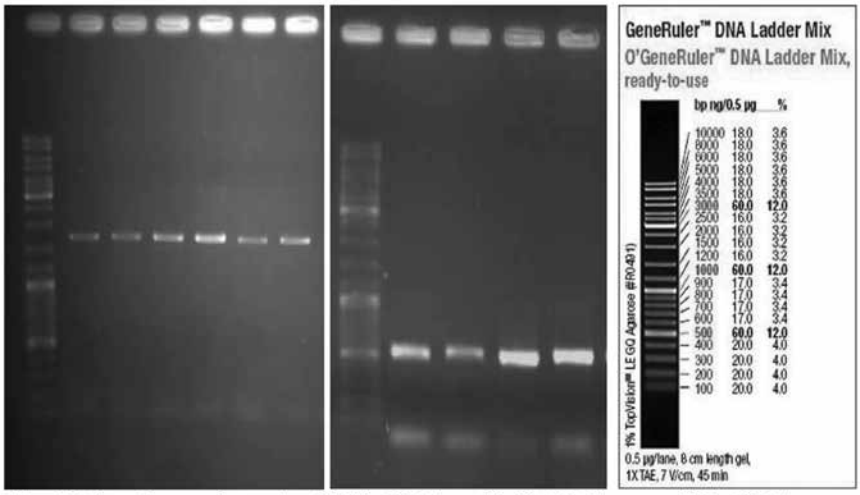

Fig. 3. The electrophoresis of 18S rRNA and 16S rRNA gene (18srRNA gene: 1800 b[illegible]

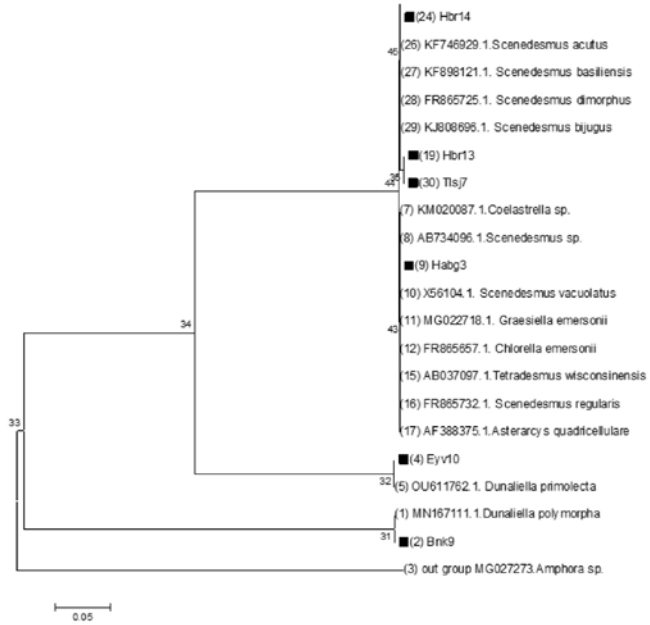

Fig. 4. The phylogenetic tree of identified strains in Eyv10, Hbr13, Hbr14, Tlsj7, Bnk9 and Habg3

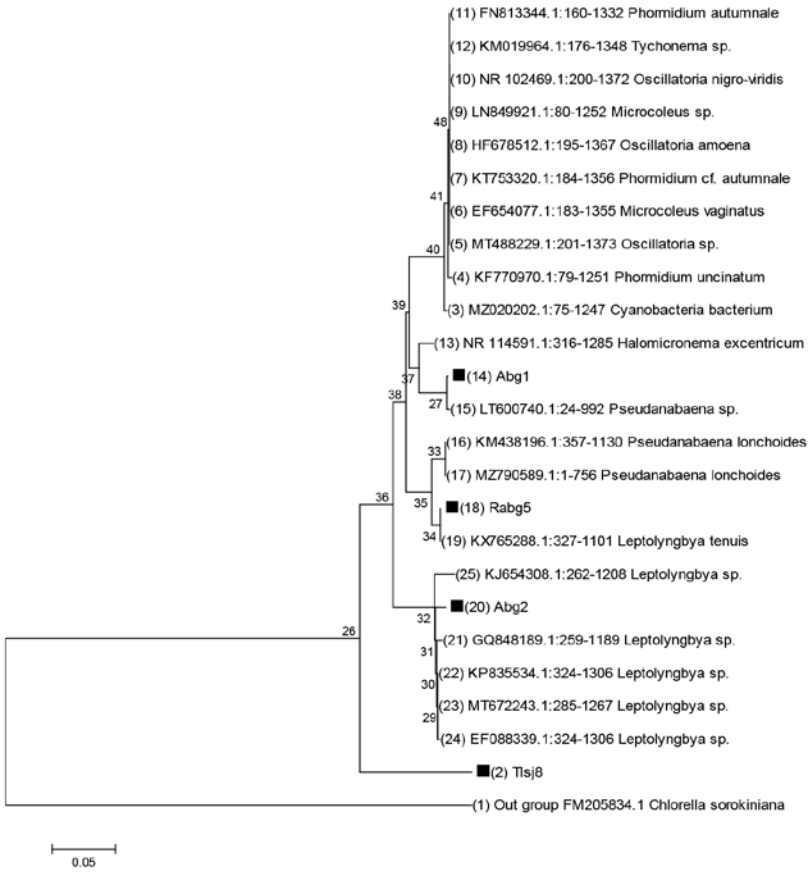

Fig. 5. The phylogenetic tree of strains in Abg1, Abg2, Tlsj8, and Rabg5

Table 5. designation and identification of strains with similarity percentage

Codes	scientific names	similarity percentage
Abg1	*Pseudanabaena* sp.	99/48% Identity and 100% Coverage
Abg2	*Leptolyngbya* sp.	98/2% Identity and 99% Coverage
Rabg5	*Leptolyngbya tenuis*	100% Identity and 100% Coverage
Tlsj8	*Leptolyngbya* sp.	89/5% Identity and 88% Coverage
Habg3	*Scenedesmus vacuolatus*	99/7% Identity and 100% Coverage
Tlsj7	*Scenedesmus obliquus*	99/8% Identity and 98% Coverage
Bnk9	*Dunaliella* sp.	93/43% Identity and 92% Coverage
Eyv10	*Dunaliella* sp.	100% Identity and 99% Coverage
Hbr13	*Scenedesmus obliquus*	99/7% Identity and 98% Coverage
Hbr14	*Scenedesmus* sp.	100% Identity and 99% Coverage

Table 6. Absorption of standard solution concentrations

concentration	absorption
125	0.326
250	0.593
500	0.839
750	1.2
1000	1.5

curves are relevant for understanding and quantifying the adsorption capacity of the tested samples.

The absorption of the total amount of antioxidants in diluted samples from each strain was measured by repeating three times at 593 nm. The average absorption results of each strain were obtained after using the formula of their standard line equation according to Table 7. After two months, the biomass of Habg3, Abg2, and Tlsj7 changed from green to orange and brown. Then their total antioxidant value was measured and named Habg33, Abg22, and Tlsj77. The results of comparing the antioxidants activity in each of the strains are shown in Figures 7 and 8.

Discussion

Several floristic studies have been carried out in different regions of Iran to identify molecular and morphological characteristics of microalgae and cyanobacteria (Mehrani Adl et al., 2020, Saba et al., 2016). This paper investigated the biodiversity of cultivable algae in the unusual waters of the Semnan and Garmsar region and isolated 10 strains. The results indicate that the Chlorophyta and Cyanophyta Phyla are more frequent than other Phyla. The high salinity sites of Bon Kooh and Iwanaki have only one *Dunaliella* sp. is isolated. *Dunaliella* sp. plays a role in the production of glycerol and beta-carotenoids, which are used in the pharmaceutical and food industries, and as an additive and colorant in cosmetics, antioxidants, and anti-cancer agents (Barbosa et al., 2023). This halophile strain helps us make optimal use of uncultivable and salt marsh areas in line with production and productivity. Therefore, the isolated *Dunaliella* has the potential for further studies and investigation for beta-carotene production. On the other side, the hot water fountains and Lasjerd station had the highest TDS and soluble elements (Table 1), and the resistant and compatible strains isolated from this station can be used for harsh environment research. For example, in high salinity and sulfur waters, it is possible to select the Lasjerd strains and conduct research on wastewater treatment. The Ab-Garm spring station in the Ab-Garm basin area had relatively high temperatures in addition to high salinities. From that area, *Leptolyngbya* sp. was isolated, showing that it can tolerate hot water, it can be used in further research, as heat-resistant industrial and medical enzymes. According to the database of algae in Iran, it was found that no research has been done to identify algae in Semnan province before. This province has great potential for extensive research in this area. Considering the desert nature of this province and the common hot and dry climate in Iran, investigated algae can be used as an alternative to biofertiliser and as a food source for livestock and poultry. While the isolation and identification of the cultivable algae in unconventional waters of the two regions have been addressed, other ecosystems such as soils have remained and no study has been done yet. Due to the environmental compatibility and low-cost basic requirements of these widely used

Table 7. The antioxidant content in each strain

Species	Codes of strains	Antioxidant activity µmol Fe^{+2}/ g of dry weight
Pseudanabaena sp.	Abg1	96.768
Leptolyngbya sp.	Abg2	34.204
Leptolyngbya tenuis	Rabg5	82.41
Leptolyngbya sp.	Tlsj8	72.153
Scenedesmus vacuolatus	Habg3	53.692
Scenedesmus obliquus	Tlsj7	69.24
Dunaliella sp.	Bnk9	53.52
Dunaliella sp.	Eyv10	252.32
Scenedesmus obliquus	Hbr13	78.13
Scenedesmus sp.	Hbr14	40.35
Leptolyngbya sp.	Abg22	214.17
Scenedesmus vacuolatus	Habg33	121.384
Scenedesmus obliquus	Tlsj77	167.538

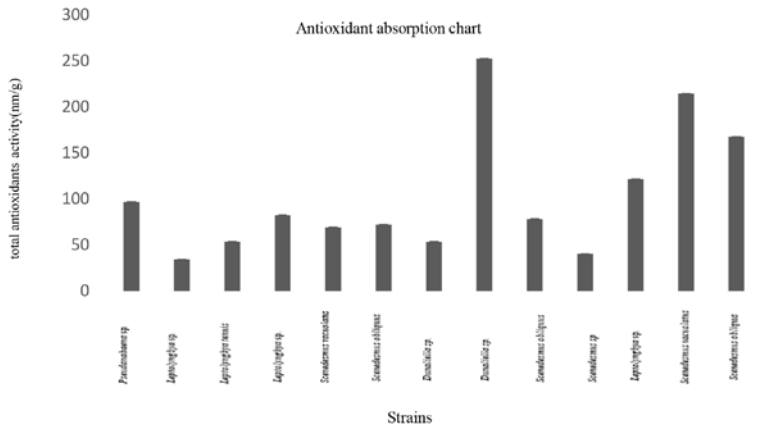

Fig.7. The total antioxidants activity in the green and red stages of algal growth

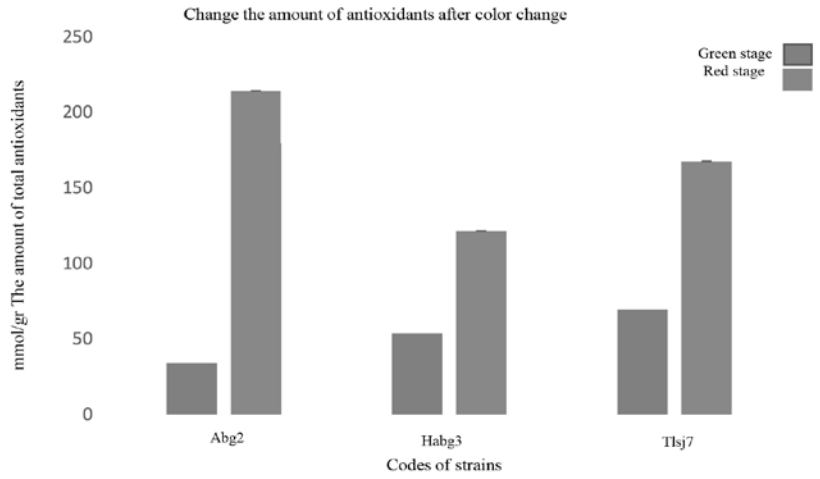

Fig. 8. The antioxidant activity in the green and red stages of algal growth

microorganisms, the growing need for the use of algae and their derived products in science and industry is well felt. Knowing this information and ensuring the genetic source, researchers can easily conduct research and economic activities.

The most important properties of algae are the antioxidant characteristics, which lead to anti-cancer, anti-hypertensive, cholesterol-lowering, immune-improving, anti-atherosclerotic, and hypoglycemic capabilities. In this study, in addition to screening and identifying isolates in designated areas, the total antioxidant content of each isolate was also evaluated.

The FRAP is a typical SET-based method for antioxidant activity assay based on the reduction of the complex of ferric ions to ferrous. Bulent et al. (2019) investigated the antioxidant activity by different methods and showed that the order of the antioxidant values of the solvent extracts measured in the FRAP method is Methanol, Ethanol, Ethyl acetate, Acetone, and then Acetonitrile. Therefore, according to the FRAP antioxidant measurement and Bluent order, the highest antioxidant activity was observed in methanol extract.

The study findings indicate that Eyv10 (*Dunaliella* sp.) exhibited the highest level of total antioxidants compared to the other strains. Following that, Abg1 (*Pseudanabaena* sp.) and Rabg5 (*L. tenuis*) displayed relatively high levels of antioxidants. In the case of Abg2 (*Leptolyngbya* sp.), the amount of antioxidants increased sixfold after the cells changed color from green to orange. This phenomenon occurs as the algae grow and produce more carotenoids, resulting in a visible color change. The orange coloration makes Abg2 suitable for extracting higher amounts of carotenoids.

Habg3 (*S. vacuolatus*) and Tlsj7 (*S. obliquus*) demonstrated nearly doubled total antioxidant levels. Notably, the color change to orange or brown significantly impacted the amount of antioxidants in these three strains. This can be attributed to the increased presence of carotenoids, which are responsible for the antioxidant properties and contribute to the observed cell color change.

The study successfully obtained and cultivated all 10 strains from Semnan province, marking the first time they have been isolated and cultured. These strains have been properly stored and officially registered in the National Center for Genetic and Biological Resources of Iran (IBRC).

Additional investigations can be conducted on strains with elevated levels of total antioxidants to evaluate their efficacy in biotechnology and industry, as well as their potential in disease treatment. Developed nations consider cancer as the primary cause of mortality, while it ranks as the second leading cause of death in developing countries. Hence, further exploration into the anticancer properties of algae antioxidants holds the potential to provide remedies for numerous illnesses. It is also advisable to undertake comprehensive examinations of diverse antioxidant agents, such as phenolic

compounds, carotenoids, and pigments, to propose future research projects.

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