Journal of Phycological Research

Vol. 5, No. 2, Sep 2021

Table of Contents

Optimization of Culture Medium to Increase The Biomass Production of *Scenedesmus obliquus*: The Impact of Carbon Source

Afsaneh Mohkami

Received: 2021-05-20 Revised and accepted: 2021-07-16

Abstract

This study aimed to investigate the effect of adding an exogenous carbon source on the growth of *Scenedesmus obliquus*. For this purpose, the impact of three carbon sources, including sodium acetate (two concentrations of 0.01 and 0.02 g/l), sodium carbonate (two concentrations of 0.01 and 0.02 g/l), and aeration to supply carbon dioxide on the growth and production of *S. obliquus* biomass cultured on BBM medium were investigated. Based on the results, all three carbon treatments significantly increased biomass production and microalgae growth compared to the control group ($P < 0.01$). Among the carbon sources studied, the concentration of 0.02 g/l sodium acetate had the most significant effect on microalgae growth. Therefore, this treatment uses for the heterotrophic culture of *S. obliquus*. Aeration treatment also increased the biomass concentration of the microalgae compared to control ($P<0.01$). This assay showed that *S. obliquus* was capable of heterotrophic growth using acetate as a carbon source. The results also showed that carbon treatments significantly increased the content of photosynthetic pigments in microalgae ($P < 0.01$). Since pH may affect algal growth, an examination of the pH trend showed that acetate and carbonate treatments significantly increased the pH of the culture medium at the end of the experiment. In contrast, aeration treatment did not affect pH change compared to the control group.

Keywords: Aeration, Sodium acetate, Heterotrophic growth, microalgae, photosynthetic pigments.

Introduction

Since the advent of algal culture medium formulations, often since the 1950s efforts have been made to optimize culture media for achieving maximum biomass (Hughes et al., 1958; Kiyohara et al., 1960). Algae culture medium is a combination of salts of macro and microelements essential for the survival and growth of algal cells. Optimization of the culture medium allows researchers to reach quickly high levels of biomass, which is an important issue both in laboratory research and in the large-scale culture of algae for commercial purposes (Verma et

Research and Technology Institute of Plant Production, Shahid Bahonar University of Kerman, Kerman, Iran
*Email: amohkami@uk.ac.ir

al., 2020). The trend of the evolution of algal culture medium in recent decades confirms the claim that culture medium optimization is an essential concern in the biotechnology of microalgae (Karpagam et al., 2015). On the other hand, aquaculture of microalgae requires a culture medium that incorporates a combination of technical, economic, and environmental considerations; highlighting the significance of ingredients in the media (Khan et al., 2018).

Carbon source is one of the main components of the culture medium that significantly impacts the growth of microalgae cells. Like all photosynthetic organisms, algae need carbon (often in the form of carbon dioxide) to grow. No change occurs in the absence of carbon, and carbon deficiency is an important limiting factor in the growth of microalgae (Li et al., 2020). On the other hand, empirical evidence shows that carbon enrichment in the culture medium significantly improves the growth and speeds up biomass production both in the laboratory and large-scale culture (Jafari et al., 2021). Although CO_2 is often used as a carbon source for algal growth, this approach suffers from shortcomings. Based on the results of the analysis of the chemical composition of the biomass of microalgae, it is determined that to produce each gram of microalgae, 1.8 grams of carbon dioxide is needed. Therefore, the use of carbon dioxide in the air alone is not enough to achieve high levels of microalgae biomass (Sydney et al., 2010). On the other hand, in most laboratory studies that require sterile conditions, aeration may lead to contamination of the culture medium (Cordoba-Perez M and de Lasa, 2020). The use of air filters to prevent contamination of the culture medium also faces technical and handling problems. Therefore, the use of a carbon source formulated as part of the culture medium is an effective way to increase microalgae biomass and at the same time, prevent contamination in aseptic cultures. Accordingly, several studies have been conducted to find a suitable carbon source for microalgae cultivation (Gim et al., 2016; Le Gouic et al., 2021).

Scenedesmus obliquus is a green microalgae species widely used to generate bio-hydrogen and biodiesel (Agrawal and Verma, 2022).

Other applications of *S. obliquus* include, and are not limited to, phycoremediation of wastewater and production of fish meal (Patnaik et al., 2019). BBM (Bold Basal Medium) is one of the major culture media for *Scenedesmus* culture with no carbon source, which encourages photoautotroph growth of microalgae. Photoautotrophic culture of microalgae suffers from limitations such as low biomass accumulation; thus, *Scenedesmus* grows more slowly than many common microalgae such as *chlorella* sp. and *chlamydomonas reinhardtii* (Matsudo et al., 2017). Accordingly, enhancing the usual cultivation media of *S. obliquus*- such as BBM - with a carbon source that promotes heterotrophic growth has the potential to have a positive effect on the growth rate and biomass production of this microalga (Wang et al., 2015). In addition, heterotro-

phic growth has advantages such as better culture control, no need for continuous aeration, and increased biomass and lipid production (El-Sheekh et al., 2013). According to these propositions, the present study was conducted to modify BBM culture medium by adding a carbon source and investigating its effect on the concentration of *S. obliquus* biomass. Aeration treatment can increase the growth of microalgae by injecting carbon dioxide- a carbon source- from air into the culture medium. In large-scale cultural settings, aeration treatment is also widely used to supply carbon and improve the growth of microalgae (Zhu et al., 2017). Therefore, aeration was also applied as a carbon source in this research to monitor its impact on microalgae growth.

Material and methods

Microalgae culture and carbon treatments

Seed culture of *S. obliquus* (IBRC-M 50028) was provided by the Iranian Biological Resource Center and used for cultivation under laboratory conditions. The microalgae were cultivated in Bold Basal Medium (BBM) manually prepared according to standard guidelines (Vonshak, 2017). *S. obliquus* cells were grown under white light with a lux intensity of 80.5 µmol/s/m^2, the temperature was kept constant at 25°C, and the pH of BBM medium was set at 6.8. The automatic timer is at 14:10 (L/D). All cultures included 250ml of BBM, which added 50 ml of fresh microalgal; the initial biomass concentration was 0.04 g/L for each experiment (Abomohra et al., 2014).

Carbon treatments included a source of Sodium acetate ($C_2H_3NaO_2$) at two concentrations (0.01 and 0.02 g/l); 2) and Sodium carbonate (Na_2CO_3) in two concentrations (0.01 and 0.02g/l); 3) without aeration and aeration to supply CO_2 to the culture medium. All cultivation procedures and carbon treatments were performed under aseptic conditions and laminar airflow. Another set of group 0.02 g/L Sodium acetate was cultivated in the dark for heterotrophic growth, and the rest conditions were the same as the above.

Growth and physiological Measurements

According to the method proposed by Almomani and Mermeci (2018), to measure the fresh weight of algae, 50 ml of culture medium was added to the Falcon tube and then centrifuged. The supernatant was discarded entirely. Falcons were then measured with a digital scale. The difference between the weight of the empty Falcon and the Falcon containing algal pellets was reported as wet weight. The Falcons were placed in a 60 °C oven for 60 hours until the algal bullet was completely dry to determine the dry weight. The Falcons were then weighed again. The difference in weight of the falcons before and after placement in the oven was reported as dry weight.

Algal growth was observed by measuring light absorption at 680 nm. The pH of the culture medium was measured with a table-top pH meter. Chlorophylls content was determined by the method proposed Gim et al. (2016). For this purpose, 10 ml of the homogeneous algal suspension was first

centrifuged at 380 g for 10 minutes. The supernatant was then discarded. Then, 5 ml of 80% acetone was added to the precipitate and then extracted for 15 minutes at 10 °C. The concentration of chlorophylls in the extracted solution was determined by measuring the absorbance at 645 and 663 nm with a UV–visible spectrophotometer (NanoDrop One UV-Vis Spectrophotometer, USA) and then calculating with the following equation:

Chlorophylls(mg/L)=8.02×A_{663}+20.21× A_{645}.

This research was carried out in a completely randomized design with three replications. Duncan's mean comparison test was performed using SAS. 10 software for statistical analysis.

Results

The present study was carried out for 14 days. The measurement results at the end of the experiment are presented in Table 1.

The biomass production at sodium acetate concentrations of 0.01 g/l and 0.02 g/l were 0.589 and 0.704 g/l; respectively, significantly higher than the control group's ($p<0.01$). The biomass production at sodium carbonate concentrations of 0.1 g/l and 0.02 g/l were 0.305 and 0.372 g/l, respectively, significantly higher than that of the control group but lower than biomass produced under aeration treatment (0.601 g/l). Overall, the results obtained concerning biomass productivity under different treatments showed that sodium acetate at a concentration of 0.02 g/l had the best results, followed by aeration, sodium acetate (0.01 g/l), and sodium carbonate (0.02 g/l), sodium carbonate (0.01), and control; respectively.

To evaluate the heterotrophic growth potential of *S. obliquus* using acetate as a carbon source, the optimum acetate concentration (0.02 g/l) in mixotrophic culture was adopted for cultivation under dark conditions. The result showed that *S. obliquus* could grow under darkness in the presence of carbon acetate at a concentration of 0.02 g/l. At the end of the experiment (14th day), a biomass concentration of 0.158 g/l was achieved. Although this value is lower than those obtained under every mixotrophic treatment and there was no significant difference with

Table 1. Summary of measurements at the end of experiment (the day 14th)

	$C_2H_3NaO_2$ (g/l)		Na_2CO_3 (g/l)				Dark
	0.01	0.02	0.01	0.02	Aeration	Control	$C_2H_3NaO_2$ 0.02 (g/l)
Biomass (g/l)	0.589	0.704	0.305	0.372	0.601	0.149	0.158
OD_{680}	1.4	1.81	0.66	084	1.5	0.42	0.43
Chlorophyll a (mg/l)	4.06	4.58	3.31	3.53	4.11	1.32	0.52
Chlorophyll b (mg/l)	1.35	1.55	1.1	1.17	1.4	0.44	0.21
Carotenoid (mg/l)	0.63	0.77	0.54	0.59	0.71	0.21	0.17
pH	10.05	11.26	10.16	11.08	8.88	7.5	8.1

control, it is of great importance to observe the adequacy of carbon acetate for heterotrophic growth of *S. obliquus*.

Optical density is an indicator for evaluating the growth of microalgae. For this purpose, the algae growth rate was measured based on the OD_{680} value on days 0, 5, 10, and 14 (Figure 3). As the trend in the chart shows, after a 24-hour lag phase, the growth graph of carbon treatments started to rise. As observed for biomass, at the end of the experiment (day 14) the highest OD levels were observed in the treatments of sodium acetate, aeration, sodium carbonate, and finally the control group, respectively. Optical density values followed the same trend as biomass values.

Regarding photosynthetic pigments, sodium acetate treatment significantly increased the content of chlorophyll a, chlorophyll b, and carotenoids compared to the control ($p<0.01$). Similarly, the increase in photo-

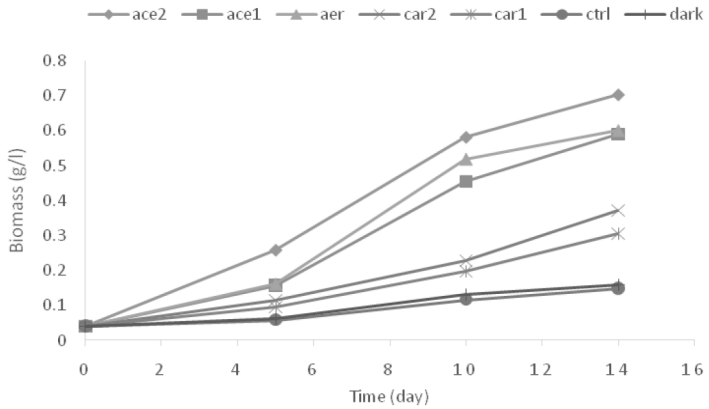

Fig. 1. Biomass concentration of *S. obliquus* under various treatments of carbon (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

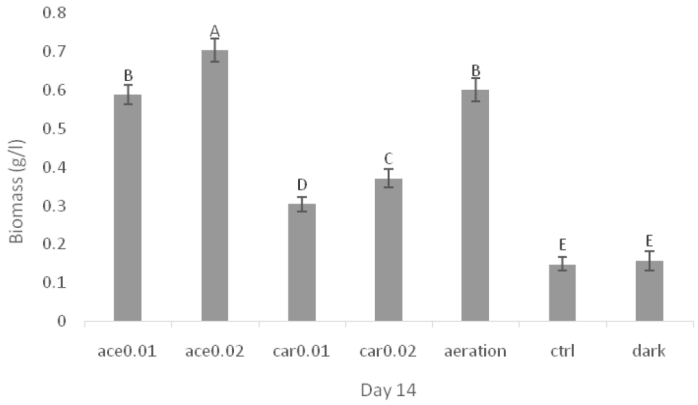

Fig. 2. Comparison of biomass production under various carbon treatments at day 14. (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02 g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

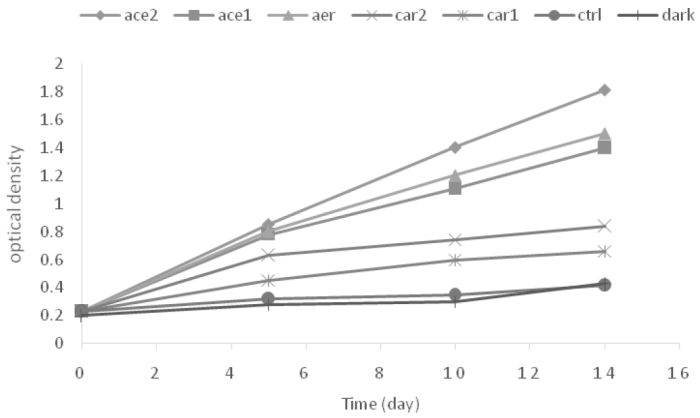

Fig. 3. Growth kinetics of *S. obliquus* under various treatments of carbon (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

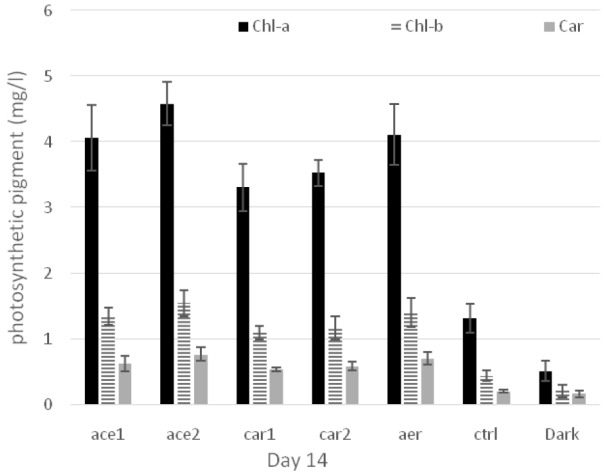

Fig. 4. Photosynthetic pigment concentration at various carbon treatments (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively)

synthetic pigments in sodium acetate treatment at 0.02 g/l was significantly higher than in other treatments. Also, aeration and sodium carbonate treatments (as a carbon source) significantly increased the content of photosynthetic pigments compared to the control group ($p<0.01$). In photosynthetic pigments, sodium acetate, aeration, and sodium carbonate treatments had the most significant effect on chlorophyll content, and increased chlorophyll b and carotenoids were in the next rank. Overall, the results of this assay showed that the use of an exogenous carbon source significantly increases photosynthetic pigments ($p<0.01$). The pH level at heterotrophic condition was also higher than that of control group under mixotrophic culture.

Microalgae have recently attracted considerable interest worldwide due to their exten-

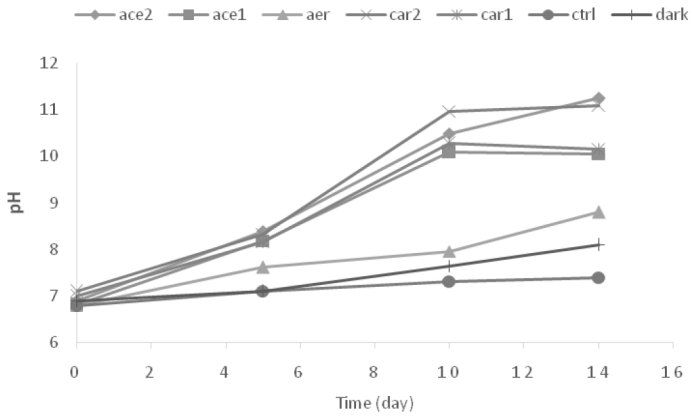

Fig. 5. pH variation as the result of carbon treatments (ace2, ace1, aer, car2, car1 and ctrl stand for 0.02g/l acetate, 0.01g/l acetate, aeration, 0.02g/l carbonate, 0.01g/l carbonate, control; respectively).

sive application potential in the renewable energy, biopharmaceutical, and nutraceutical industries. Microalgae are renewable, sustainable, and economical sources of biofuels, bioactive medicinal products, and food ingredients (Verma et al., 2020). Microalgae are mainly photoautotrophs and grow in a culture medium containing mineral salts; because microalgae can convert sunlight into chemical energy. However, autotrophic culture can't provide sufficient efficiency to produce microalgae biomass. Autotrophic culture increases production costs due to the constant need for light and technical considerations in the design of bioreactors (Lu et al., 2021). Therefore, switching to heterotrophic culture - which uses a carbon source - has been proposed as a suitable solution to increase the biomass production efficiency of microalgae. Some microalgae species, such as *S. obliquus*, can grow heterotrophically or mixotrophically (Cordoba-Perez et al., 2020).

Discussion

One of the main components of heterotrophic culture systems is using an external carbon source to meet the metabolic needs of microalgae. Therefore, the design of carbon source-based heterotrophic systems and determining the optimal concentration of carbon source to achieve a cost-effective level of biomass in such algae are of great importance (Gim et al., 2016, Jafari et al., 2021).

One of the remarkable results was that the amount of biomass produced with aeration is very close to sodium acetate, which shows the critical role of aeration. Because aeration is a cost-effective and easy method, this treatment can be used as a source to supply carbon to the microalgae culture medium. Previous studies have also highlighted the importance of aeration to improve the growth of microalgae. For example, Tang et al. (2016) found that aeration treatment enhances the development of both microorganisms (algae and bacteria) in a survey of

an algal-bacterial symbiosis system. Similarly, Liu et al. (2006) showed that artificial aeration has a positive effect on microalgae growth; these results support the findings of the present study.

Based on the results obtained in the present study, *S. obliquus* microalgae can grow mixotrophically in the presence of a carbon source in cultures containing acetate and carbonate. Even the microalgae could undergo heterotrophic growth when supplied with carbon acetate. This finding is of great practical importance, autotrophic and inability to heterotrophic growth have been considered limiting factors in algal biomass production in many studies (Scarponi et al., 2021). Heterotrophic growth significantly reduces cultivation costs and increases productivity (Karpagam et al., 2015). Heterotrophic culture is of great importance in producing microalgae biomass for several reasons. One of the biggest problems in autotrophic cultivation is the lack of light reaching the depths of the culture medium, which causes a significant drop in biomass production efficiency (Morales-Sánchez et al., 2015).

On the other hand, the phenomenon of light saturation is considered a factor is inhibiting the growth of microalgae in autotrophic culture. Problems of autotrophic cultivation are significant in the large-scale cultivation of microalgae because it reduces microalgae biomass production units (Ye et al., 2018). Many researchers consider many researchers have viewed the use of heterotrophic culture. First, in heterotrophic culture, more control is possible and thus can reduce the amount of potential contamination of the culture medium. Moreover, there is no need for continuous aeration in heterotrophic cultivation, which plays a vital role in reducing energy consumption and production costs (El-Sheekh et al., 2013).

Numerous reports have also suggested that the production of biomass and lipids can be increased in heterotrophic cultures (Sutherland et al.., 2021; Senet al. l, 2018). Accordingly, the ability of microalgae to heterotrophic growth is both technically and economically significant.

On the other hand, it has already been mentioned that one of the main challenges in the large-scale production of algal biomass is due to the inability of algal species to heterotrophic growth (El-Sheekh et al., 2013). Another finding of the present study is a significant increase in biomass production as a result of the application of carbon sources (sodium acetate and sodium carbonate). Because *S. obliquus* is a species with high commercial value, improving biomass production efficiency can be of great economic importance. Efforts have already been made to increase biomass production in *S. obliquus*. For example, Mandal and Mallick (2009) studied a combination of different factors to increase the biomass of *S. obliquus* and found that using an appropriate light regime and a combination of other minerals has a significant effect on increasing the growth of this microalgae. Abomohra et al. (2014) reported that temperature optimization and growth regulators significantly improved *S. obliquus* biomass. De Oliveira et

al. (2020) introduced the improvement of biomass production as a vital factor in the commercialization of *S. obliquus* cultivation. In general, the literature indicates the high importance of biomass production optimization methods for the large-scale cultivation of *S. obliquus*.

Since microalgae such as *S. obliquus* are photosynthetic organisms, studying their photosynthetic apparatus under different treatments is of great importance. For this purpose, in the present study, the effect of carbon treatments on photosynthetic pigments of *S. obliquus* was investigated. Based on the results, it was found that all three treatments of sodium acetate, sodium carbonate, and aeration significantly increased chlorophylls and carotenoids in microalgae. These findings indicate that using an external carbon source is a desirable factor to improve the growth and stimulate photosynthetic activity in microalgae. The results obtained in this assay are consistent with the findings reported by other authors. For example, Song & Pei (2018) said that using an exogenous carbon source increases the content of chlorophyll and carotenoids in the microalgae *Scenedesmus quadricauda*. Pancha et al. (2015) pointed out that optimizing the carbon composition in *Scenedesmus* sp. culture medium significantly increases photosynthetic pigments. Similarly, Vijay et al. (2021) reported that the *S. obtusus* could efficiently utilize the supplemented carbon source, increasing its photosynthetic efficiency.

One of the main components of heterotrophic culture systems is using an external carbon source to meet the metabolic needs of microalgae. Therefore, the design of carbon source-based heterotrophic systems and determining the optimal carbon source concentration to achieve a cost-effective biomass level in such algae are of great importance (Gim et al., 2016; Jafari et al., 2021). One of the remarkable results was that the amount of biomass produced with aeration is very close to sodium acetate, which shows the critical role of aeration. Because aeration is a cost-effective and easy method, this treatment can be used as a source to supply carbon to the microalgae culture medium. Previous studies have also highlighted the importance of aeration to improve the growth of microalgae. For example, Tang et al. (2016) found that aeration treatment enhances the development of both microorganisms (algae and bacteria) in a survey of an algal-bacterial symbiosis system. Similarly, Liu et al. (2006) showed that artificial aeration positively affects microalgae growth; these results support the present study's findings.

Based on the results obtained in the present study, *S. obliquus* microalgae can grow mixotrophically in the presence of a carbon source in cultures containing acetate and carbonate. Even the microalgae could undergo heterotrophic growth when supplied with carbon acetate. This finding is of great practical importance; autotrophic and inability to heterotrophic growth have been considered limiting factors in algal biomass production in many studies (Scarponi et al., 2021).

Heterotrophic growth significantly reduces cultivation costs and increases productivity (Karpagam et al., 2015). Heterotrophic culture is of great importance in producing microalgae biomass for several reasons. One of the biggest problems in autotrophic cultivation is the lack of light reaching the depths of the culture medium, which causes a significant drop in biomass production efficiency (Morales-Sánchez et al., 2015).

On the other hand, the phenomenon of light saturation is considered a factor is inhibiting the growth of microalgae in autotrophic culture. Problems of autotrophic cultivation are significant in the large-scale cultivation of microalgae because it reduces microalgae biomass production units (Ye et al., 2018). Many researchers consider many researchers have viewed the use of heterotrophic culture. First, in heterotrophic culture, more control is possible and thus can reduce the amount of potential contamination of the culture medium. Moreover, there is no need for continuous aeration in heterotrophic cultivation, which plays a vital role in reducing energy consumption and production costs (El-Sheekh et al., 2013).

According to the results of this study, sodium acetate as a carbon source had the most significant effect on increasing the growth of *S. obliquus*. This finding suggests that sodium acetate can provide sufficient carbon for microalgae's growth and metabolic processes, thereby increasing its biomass. Some previous studies have also suggested the importance of sodium acetate as a carbon source for the development of microalgae. For example, enrichment of BBM medium with 7.5 g/l each of glucose and sodium acetate has been reported to produce dry biomass equal to 1.75 g/l in the microalgae *Neochloris oleoabundans* (Silva et al., 2016). Similarly, Wang et al. (2019) demonstrated that Coccomyxa subellipsoidea subjected to the feeding of 12 g/l sodium acetate achieved favorable biomass of 1.35 g/l and lipid content of 52.16%. The results obtained in this study are consistent with the results of other researchers on the adequacy of sodium acetate as a carbon source for the growth of microalgae. For example, Lacroux et al. (2021) showed that using sodium acetate with a nitrogen source significantly improved the development and production of biomass in *Chlorella sorokiniana.*

Similarly, Turon et al. (2015) showed that acetate is a good carbon source for the heterotrophic growth of microalgae. Jeon et al. (2006) also showed that the combination of light intensity and sodium acetate as a carbon source has a significant effect on the growth of *Haematococcus pluvialis*. Although these studies demonstrate the importance of acetate as a carbon source in the heterotrophic growth of microalgae, acetate has been studied less concerning with *S. obliquus*. The literature shows that researchers have explored other carbon sources in heterotrophic growth.

In previous studies, other carbon sources other than sodium acetate or sodium carbonate have been introduced as promoting factors for growth and biomass production in *S. obliquus*. Yang et al. (2014) reported

the utility of xylose as a carbon source for the mixotrophic growth of *S. obliquus*. Matsudo et al. (2017) investigated ethanol as a complementary carbon source in *S. obliquus* cultivation. They concluded that alcohol has a positive impact on biomass production of the microalgae. More recently, Scarponi et al. (2021) recommended the application of organic solid waste digest as the carbon source for the growth and cultivation of *S. obliquus*. Abomohra et al. (2014) called for introducing a new and cost-effective alternative for large-scale commercial cultivation of *S. obliquus*. According to our results, in addition to acetate, sodium carbonate as a carbon source was able to improve the growth of *S. obliquus* significantly. Since sodium carbonate is a relatively inexpensive carbon source that can use for the heterotrophic growth of *S. obliquus* microalgae, some previous studies also show that sodium carbonate is sufficient to supply the carbon required to develop microalgae (Elvira-Antonio et al., 2013; Duan et al., 2020). The results are consistent with the findings of the present study.

References

Abomohra AEF, El-Sheekh M, Hanelt D. (2014). Pilot cultivation of the chlorophyte microalga *Scenedesmus obliquus* as a promising feedstock for biofuel. Biomass and Bioenergy. 64: 237-244.

Agrawal K and Verma P. (2022). An overview of various algal biomolecules and its applications. An Integration of Phycoremediation Processes in Wastewater Treatment. 249-270. Doi: 10.1016/B978-0-12-823499-0.00006-7.

Almomani FA and Örmeci B. (2018). Monitoring and measurement of microalgae using the first derivative of absorbance and comparison with chlorophyll extraction method. Environmental monitoring and assessment, 190 (2): 1-19.

Cordoba-Perez M and de Lasa H. (2020). CO_2-Derived carbon capture and photon absorption efficiency by microalgae in novel photo bio CREC. Industrial & Engineering Chemistry Research. 59 (33): 14710-14716. Doi:10.1021/acs.iecr.0c02319.

de Oliveira, CYB, Viegas, TL, Lopes RG, Cella H, Menezes RS, Soares AT, Derner RB. (2020). A comparison of harvesting and drying methodologies on fatty acids composition of the green microalga *Scenedesmus obliquus*. Biomass and Bioenergy. 132, 105437.

Duan Y, Guo X, Yang J, Zhang M, Li, Y. (2020). Nutrients recycle and the growth of *Scenedesmus obliquus* in synthetic wastewater under different sodium carbonate concentrations. Royal Society open science. 7 (1): 191214.

El-Sheekh MM, Hamouda RA, Nizam AA. (2013). Biodegradation of crude oil by *Scenedesmus obliquus* and *Chlorella vulgaris* growing under heterotrophic conditions. International Biodeterioration & Biodegradation. 82: 67-72.

Elvira-Antonio N, Ruíz-Marín A, Canedo-López Y. (2013). Effect of nitrogen content and CO_2 consumption rate by adding sodium carbonate in the lipid content of *Chlorella vulgaris*

and *Neochloris oleoabundans*. International Journal of Environmental Protection. 3 (10): 13.

Gim GH, Ryu J, Kim MJ, Kim PI, Kim SW. (2016). Effects of carbon source and light intensity on the growth and total lipid production of three microalgae under different culture conditions. Journal of Industrial Microbiology and Biotechnology. 43 (5): 605-616. Doi: 10.1007/s10295-016-1741-y.

Hughes EO, Gorham PR, Zehnder A. (1958). Toxicity of a unialgal culture of *Microcystis aeruginosa*. Canadian Journal of Microbiology. 4 (3): 225-236.

Jafari A, Esmaeilzadeh F, Mowla D, Sadatshojaei E, Heidari S, Wood DA. (2021). New insights to direct conversion of wet microalgae impregnated with ethanol to biodiesel exploiting extraction with supercritical carbon dioxide. Fuel. 285: 119199. Doi: 10.1016/j.fuel.2020.119199.

Jeon YC, Cho CW, Yun YS. (2006). Combined effects of light intensity and acetate concentration on the growth of unicellular microalga *Haematococcus pluvialis*. Enzyme and microbial technology. 39 (3): 490-495.

Karpagam R, Raj KJ, Ashokkumar B, Varalakshmi P. (2015). Characterization and fatty acid profiling in two fresh water microalgae for biodiesel production: lipid enhancement methods and media optimization using response surface methodology. Bioresource Technology. 188, 177-184. Doi:10.1016/j.biortech.2015.01.053.

Khan MI, Shin JH, Kim JD. (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial Cell Factories. 17 (1): 1-21. Doi:10.1186/s12934-018-0879-x.

Kiyohara T, Fujita Y, Hattori A, Watanabe A. (1960). Heterotrophic culture of a blue-green alga, *Tolypothrix tenuis* I. The Journal of General and Applied Microbiology. 6 (3): 176-182.

Lacroux J, Seira J, Trably E, Bernet N, Steyer JP, van Lis R. (2021). Mixotrophic growth of *Chlorella sorokiniana* on Acetate and Butyrate: Interplay Between Substrate, C: N Ratio and pH.Frontiers in microbiology. 12: 1830.

Le Gouic B, Marec H, Pruvost J, Cornet JF. (2021). Investigation of growth limitation by CO_2 mass transfer and inorganic carbon source for the microalga *Chlorella vulgaris* in a dedicated photobioreactor. Chemical Engineering Science. 233: 116388. Doi:10.1016/j.ces.2020.116388.

Li S, Ji L, Chen C, Zhao S, Sun M, Gao Z, Fan J. (2020). Efficient accumulation of high-value bioactive substances by carbon to nitrogen ratio regulation in marine microalgae *Porphyridium purpureum*. Bioresource Technology. 309: 123362. Doi: 10.1016/j.biortech.2020.123362

Liu CG, Jin XC, Sun L, Sun HW, Zhu L, Yu Y, Zhuang YY. (2006). Effects of nitrogen source and aeration mode on algae growth in freshwater. *Huanjingkexue= Huanjingkexue*. 27 (1): 101-104.

Loftus SE and Johnson ZI. (2019). Reused cultivation water accumulates dissolved organic carbon and uniquely influences different marine microalgae. Frontiers in Bioengineering and Biotechnology. 7: 101. Doi: 10.3389/

fbioe.2019.00101.

Lu W, Liu S, Lin Z, Lin M. (2021). Enhanced microalgae growth for biodiesel production and nutrients removal in raw swine wastewater by carbon sources supplementation. Waste and Biomass Valorization. 12 (4): 1991-1999.

Mandal S and Mallick N. (2009). Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Applied Microbiology and Biotechnology. 84 (2): 281-291.

Matsudo MC, Sousa TF, Pérez-Mora LS, Bezerra R. P, Sato S, Carvalho JC. (2017). Ethanol as complementary carbon source in *Scenedesmus obliquus* cultivation. Journal of Chemical Technology and Biotechnology. 92 (4): 781-786. Doi: 10.1002/jctb.5059.

Morales-Sánchez D, Martinez-Rodriguez OA, Kyndt J, Martinez A. (2015). Heterotrophic growth of microalgae: metabolic aspects. World Journal of Microbiology and Biotechnology. 31 (1): 1-9.

Pancha I, Chokshi K, Mishra S. (2015). Enhanced biofuel production potential with nutritional stress amelioration through optimization of carbon source and light intensity in *Scenedesmus* sp. CCNM 1077. Bioresource Technology. 179: 565-572.

Patnaik R, Singh NK, Bagchi SK, Rao PS, Mallick N. (2019). Utilization of *Scenedesmus obliquus* protein as a replacement of the commercially available fish meal under an algal refinery approach. Frontiers in Microbiology. 10: 2114. Doi: 10.3389/fmicb.2019.02114.

Scarponi P, Ghirardini AV, Bravi M, Cavinato C. (2021). Evaluation of *Chlorella vulgaris* and *Scenedesmus obliquus* growth on pretreated organic solid waste digestate. Waste Management. 119: 235-241.

Sen R, and Martin GJ. (2018). Glycerol and nitrate utilisation by marine microalgae *Nannochloropsis salina* and *Chlorella* sp. and associated bacteria during mixotrophic and heterotrophic growth. Algal Research. 33: 298-309.

Silva HR, Prete CEC, Zambrano F, de Mello VH, Tischer CA, Andrade DS. (2016). Combining glucose and sodium acetate improves the growth of *Neochloris oleoabundans* under mixotrophic conditions. AMB Express. 6 (1): 1-11.

Song M and Pei, H. (2018). The growth and lipid accumulation of *Scenedesmus quadricauda* during batch mixotrophic/heterotrophic cultivation using xylose as a carbon source. Bioresource Technology. 263: 525-531.

Sutherland DL, Burke J, Ralph PJ. (2021). High-through put screening for heterotrophic growth in microalgae using the biolog Plate assay. New Biotechnology. 65: 61-68.

Sydney, EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Pandey A, Soccol, CR. (2010). Potential carbon dioxide fixation by industrially important microalgae. Bioresource Technology, 101 (15): 5892-5896. Doi: 10.1016/j.biortech.2010.02.088.

Tang, CC, Zuo, W, Tian Y, Sun N, Wang ZW, Zhang J. (2016). Effect of aeration rate on performance and stability of algal-bacterial symbiosis system to treat domestic wastewater in sequencing batch reactors. Bioresource Technology. 222: 156-164.

Turon V, Trably E, Fayet A, Fouill E, Steyer JP. (2015). Raw dark fermentation effluent to

support heterotrophic microalgae growth: microalgae successfully out compete bacteria for acetate. Algal Research. 12: 119-125.

Verma R, Kumari KK, Srivastava A, Kumar A. (2020). Photoautotrophic, mixotrophic, and heterotrophic culture media optimization for enhanced microalgae production. Journal of Environmental Chemical Engineering. 8 (5): 104149. Doi: 10.1016/j.jece.2020.104149.

Vijay AK, Salim SAM, Prabha S, George B. (2021). Exogenous carbon source and phytohormone supplementation enhanced growth rate and metabolite production in freshwater microalgae *Scenedesmus obtusus* Meyen. Bioresource Technology Reports. 14: 100669.

Vonshak A. (2017). Laboratory techniques for the cultivation of microalgae. In CRC handbook of microalgal mass culture (pp. 117-146). CRC Press.

Wang R, Xue S, Zhang D., Zhang Q, Wen S, Kong D, Cong W. (2015). Construction and characteristics of artificial consortia of *Scenedesmus obliquus*-bacteria for *S. obliquus* growth and lipid production. Algal Research. 12: 436-445.

Wang Z, Luo F, Wang Z, Zhou R, Tang Y, Li Y. (2019). The potential growth and lipid accumulation in *Coccomyxa subellipsoidea* triggered by glucose combining with sodium acetate. World Journal of Microbiology and Biotechnology. 35 (7): 1-13.

Yang S, Liu G, Meng Y, Wang P, Zhou S, Shang H. (2014). Utilization of xylose as a carbon source for mixotrophic growth of *Scenedesmus obliquus*. Bioresource Technology. 172: 180-185.

Ye Y, Huang Y, Xia A, Fu Q, Liao Q, Zeng W, Zhu X. (2018). Optimizing culture conditions for heterotrophic-assisted photoautotrophic biofilm growth of *Chlorella vulgaris* to simultaneously improve microalgae biomass and lipid productivity. Bioresource Technology. 270: 80-87.

Zhu J, He Y, Wang J, Qiao Z, Wang Y, Li Z, Huang M. (2017). Impact of aeration disturbances on endogenous phosphorus fractions and their algae growth potential from malodorous river sediment. Environmental Science and Pollution Research. 24 (9): 8062-8070.

Macroalgae-Derived Fungal Endophytes Promoted The Growth of Mexican Lime Seedlings Under Heat Stress in Greenhouse Condition

Leila Baghazadeh Daryaii[1], Davood Samsampour[2*], Abdoolnabi Bagheri[3], Jelveh Sohrabipour[4]

Received: 2021-06-25 Revised and accepted: 2021-09-28

Abstract

Fungal endophyte associated with algae represents a rich source of bioactive metabolites and biostimulants, which can be used practically in agriculture as biofertilizers. Here, we aimed to study the associated symbiotic fungi collected from intertidal areas of the southern coastlines of the Persian Gulf and Oman Sea. The extracted endophytic fungi were identified based on morphological, physiological and molecular (based on ITS1-5.8S-ITS4rRNA regions) analyses. 566 fungal isolates were obtained from 190 seaweed segments. Results showed that the genera *Aspergillus* and *Penicillium* were the most frequent isolated fungi. The highest frequency of fungal isolates (48.29%) was observed in seaweeds collected from Bushehr province. In vitro, most of the fungal isolates (85%) could grow properly on a PDA medium incorporated with one molar NaCl concentration. Fungal isolates showing the highest resistance to NaCl in vitro assays and with highest frequency were used as biofertilizer agents to study their effects on the morphological characteristics of Mexican lime seedlings. The inoculation results showed that the fungal endophytes could increase the fitness of Mexican lime seedlings under heat stress by improving morphological attributes.

Keywords: Macroalgae, Endophytic Fungi, Biofertilizer, *Aspergillus*, *Penicillium*

Introduction

The marine ecosystem is a unique source of natural biological products from marine creatures with outstanding natural, biochemical, and biosynthesis characteristics (Subramani and Aalbersberg, 2013; Suriya et al., 2016; Jimenez, 2018). Endophytic microorganisms are a diverse group of organisms that live inside the host plant tissue and form colonies in the intercellu-

1-Ph.D. in Horticultural Science-Biotechnology and Molecular Genetic of Horticultural Products, University of Hormozgan, Bandar Abbas, Iran
2-Horticultural Science Department, Agriculture and Natural Resources College, University of Hormozgan, Bandar Abbas, Iran
3-Plant Protection Research Department, Hormozgan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran
4-Department of Natural Resources Researches, Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran
* E-mail address: Samsampoor@hormozgan.ac.ir

lar spaces or between the xylem vessels of plants without any apparent symptoms (Rodriguez et al., 2009). Although, recently, a few studies have shown that infection by endophytes can increase the host plant's tolerance to abiotic and biotic stresses by inducing the activity of many defense-related genes of the host plants (Mejía et al., 2014; Miliute et al., 2015). Many questions remain unanswered regarding the biological attributes of endophytes (Suryanarayanan, 2013).

Algae in marine ecosystems adapt to frequent and sporadic environmental changes such as low oxygen content, high salinity, excessively high light, and nutrient limitation, which may be associated with endophytes to produce specific bioactive secondary metabolites to participate in the defense mechanisms of the hosts (Zhang et al., 2016).

Fungal endophytes constitute an inexplicably diverse group of polyphyletic fungi ubiquitous in plants and maintain an indiscernible dynamic relationship with their hosts for at least a part of their life cycle (Kusari and Spiteller, 2012). The endophytic fungi activate gene-silencing mechanisms, or vice versa, and start specific biosynthetic pathways. Hence, unique functional/bioactive metabolites will produce due to the reciprocal and advantageous relationship between endophytes and their hosts (Zhang et al., 2016). Symbiotic fungi have already been isolated from various marine habitats, including marine plants (algae and mangrove plants), aquatic invertebrates (sponges, holothurians, corals, and ascidians), and vertebrates (mainly fish). Among these organisms, algae are one of the most prevalent sources of marine-derived fungi for chemical studies (Rateb and Ebel, 2011).

Davati et al. (2013) studied the bioactive compounds extracted from endophytic fungi of macroalgae (Rhodophyta, Chlorophyta, and Phaeophyta) and reported different functions for these products. Anticancer, antibiotic, antiviral, antioxidative, and kinase inhibitory activities have been isolated from seaweed endophytic fungi (Strobel and Daisy, 2003; Kjer et al., 2010). Nevertheless, there is not much information regarding endophytic fungi in marine seaweeds, especially those are grown in tropical and subtropical regions (Suryanarayanan et al., 2010; Narayanan et al., 2013; Flewelling et al., 2015). Since no documented information is available regarding symbiotic fungi with seaweed species of the Persian Gulf and Oman Sea in the south of Iran, in the current study, we aimed to investigate the diversity of fungal endophytes of seaweeds of the Persian Gulf and Oman Sea and their role as stress-modifiers in Mexican lime seedlings under heat stress in greenhouse conditions.

Material and methods

Sampling and isolation of endophytic fungi

From April 2018 to March 2019, A total of 190 seaweed samples (37 species) belonging to 12 brown algae (62 samples), 15 red (79 pieces), and 10 green algae species

(49 pieces) were collected from 18 intertidal locations of three southern provinces of Iran including; Hormozgan, Bushehr and Sistan and Baluchistan (Table 1). Seaweeds were identified based on morphological keys (Sohrabipour and Rabiei, 2007; 2008; 2010).

The seaweed species in different sites were replicated. For example, we collected *Ulva* or *Gracilaria* from different sides and different seasons.

The physicochemical properties of seawater including EC, pH, and salinity were measured using the multimeter (HACH, HQ 40d USA) and refractometer (ATAGO, S/Mill-E Japan) devices (Table 2).

Fresh and healthy algal species were harvested and transferred to the biotechnology laboratory of Hormozgan University in sterile polyethylene bags. The surface of the samples was sterilized to isolate the exophytes from the collected seaweeds, as follows: washing the seaweeds with running tap water; soaking the illustrations in 70% ethanol for 5 S and, finally soaking in sterile distilled water for 10 S (Zhang et al., 2009). Sterilized tissues were cut into 0.5 cm^2 segments, then three segments of each seaweed sample were placed onto Petri plates containing Potato Dextrose Agar (PDA). Accordingly, 1674 sterilized segments of 190 seaweed specimens were used to isolate their endophytic fungi. The Petri plates were sealed and incubated in a light chamber at 26 ± 2 °C and a light period (12L:12D) for four weeks (Suryanarayanan, 1992). Most fungi grew within three days after incubation. The colonization frequency (CF) was calculated based on Yuan et al. (2010) as follows:

CF= total number of colonized endophytic fungi/ total number of incubated segments.

The grown fungi of each seaweed segment were periodically isolated and transferred to the fresh PDA plates. Identification of the isolated fungi was made based on morphological, physiological, and molecular methods. Microscopic characteristics of the isolates (hypha type, spore shape, and colony color) were also investigated to determine the morphological features of fungal isolates.

Molecular identification and phylogenetic analysis

DNA extraction

The fungal endophytes were identified molecularly using ITS primers (ITS1-5.8S-ITS4 region of rRNA). Genomic DNA was extracted according to the method proposed by Soltani and Moghaddam (2015). Colonies of each isolated fungi were grown at 28 °C and shaking rate of 90 rpm in the 15 ml Erlenmeyer flasks containing 2 ml Potato Dextrose Broth (PDB; Merck, Germany). After 15 days, the genomic DNA of each isolate was extracted using the CTAB method.

PCR amplification

The extracted DNA was subjected to Polymerase Chain Reaction (PCR) to amplify the ITS1-5.8S-ITS4rRNA region using the following primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)

Table 1. List of seaweed species and their classification

No	Scientific name	Classification
1	*Stoechospermum marginatum*	Phaeophyta
2	*Padina australis*	Phaeophyta
3	*Padina crassa*	Phaeophyta
4	*Padina gymnospora*	Phaeophyta
5	*Rosenvingea orientalis*	Phaeophyta
6	*Sargassum boveanum*	Phaeophyta
7	*Sargassum bov.* Var. *aterrimum*	Phaeophyta
8	*Sargassum angustifolium*	Phaeophyta
9	*Dictyota* sp.	Phaeophyta
10	*Sirophysalis trinodis*	Phaeophyta
11	*Grateloupia lithophila*	Phaeophyta
12	*Iyengaria stellata*	Phaeophyta
13	*Galaxaura rugosta*	Rhodophyta
14	*Acanthophora spicifera*	Rhodophyta
15	*Hypnea* sp.	Rhodophyta
16	*Digenenia simplex*	Rhodophyta
17	*Gracilaria folliifera*	Rhodophyta
18	*Gracillaria corticata*	Rhodophyta
19	*Gracilaria* sp.	Rhodophyta
20	*Gracilaria salicornia*	Rhodophyta
21	*Gracilariopsis persica*	Rhodophyta
22	*Palisada perforata*	Rhodophyta
23	*Champia glublifera*	Rhodophyta
24	*Champia parvulla*	Rhodophyta
25	*Jania robens*	Rhodophyta
26	*Laurencia snideria*	Rhodophyta
27	*Grateloupia flicina*	Rhodophyta
28	*Halimeda tuna*	Chlorophyta
29	*Caulerpa sertularioides*	Chlorophyta
30	*Caulerpa peltata*	Chlorophyta
31	*Caulerpa taxifolia*	Chlorophyta
32	*Cladophoropsis* sp.	Chlorophyta
33	*Ulva ohnii*	Chlorophyta
34	*Ulva* sp.	Chlorophyta
35	*Chondrus crispus*	Chlorophyta
36	*Chaetomorpha* sp.	Chlorophyta
37	*Enteromorpha* sp.	Chlorophyta

Table 2. List of provinces, locations and their GPS coordinates as well as chemical properties of seawater in different sampling sites

Province	Location	Longitude (E)	Latitude (N)	EC (ms/cm)	pH	Salinity (PPT)
Hormozgan	Bandar Abbas	E56.27	N27.18	56.9	7.49	39.3
Hormozgan	Bandar-e Lengeh	E54.30	N26.18	56.6	7.97	38.4
Hormozgan	Bandar-e Kong	E54.54	N29.33	57.9	7.99	39.6
Hormozgan	Qeshm Island	E56.16	N26.57	56.6	7.94	38.9
Hormozgan	Hormoz Island	E56.27	N27.03	56.5	7.52	39.0
Hormozgan	Sirik	E57.10	N26.51	54.5	7.44	38.2
Sistan and Baluchistan	Chabahar	E60.39	N25.17	52.8	8.1	37.6
Sistan and Baluchistan	Gavader	E61.31	N25.10	54.5	7.12	36.9
Sistan and Baluchistan	Ramin	E60.25	N25.12	56.3	7.87	38.6
Sistan and Baluchistan	Great see	E60.39	N25.16	55.4	8.13	38.2
Sistan and Baluchistan	Kachu	E60.51	N25.15	56.4	8.2	38.1
Sistan and Baluchistan	Beris	E61.11	N25.08	55.5	7.98	38.3
Sistan and Baluchistan	Konarak	E60.23	N25.21	56.4	7.88	38.9
Bushehr	Bushehr	E50.83	N28.95	62.7	8.27	**42.0***
Bushehr	Shif Island	E50.52	N29.04	49.8	7.74	33.9
Bushehr	Nuclear Power plant	E50.53	N28.49	60.5	8.06	**41.0**
Bushehr	Sabz Abad park	E50.51	N28.44	60.2	8.06	**41.0**
Bushehr	Airport seaside	E50.50	N28.56	49.9	7.80	33.9

*Samples with ≥40 PPT salinity, were detected by refractometer device

(White et al., 1991). Each 25 µl reaction mixture included 2 µl of DNA, 12.5 µl of Taq DNA Polymerase (amplicon), 2 µl of primers, and 8.5 µl ddH_2O. Techno TC-572 thermocycler (Eppendorf, Hamburg, Germany) was used for PCR amplification which was programmed for 94 °C for 5 min, 35 cycles of 94 °C for 45 S, 53 °C for 30 S, 72 °C for 1 min, and 72 °C for 5 min. PCR products were subjected to electrophoresis on 1% agarose gel stained with blue stain.

DNA sequencing

All PCR products were sequenced directly by Macrogen Sequencing Service (Seoul, South Korea). The sequences of amplified ITS1-5.8S-ITS4rRNA regions were deposited in the GenBank database (NCBI, http://www.ncbi.nlm.nih.gov). The edited sequences were blasted against the NCBI GenBank nucleotide database (http://ncbi.nlm.nih.gov/blast/Blast.cgi) to search for closest relatives. Phylogenetic tree was reconstructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The fungal isolates were archived as living vouchers at 4 °C.

Fig. 1. Fungal inoculum preparation using autoclaved water in 1×10^6 CFU mi^{-1} colony formation

Potential of endophytic fungi to grow under salt conditions

The potential of fungal endophytes to grow under salt conditions were investigated using the method proposed by Vatsyayan and Ghosh (2013). In brief, fungal isolates were grown in PDA medium supplemented with various concentrations of sodium chloride (1, 2 and 3 molar) and after 7 days their growth potential was scored from 1 to 8 (from weak to very severe), which was based on their ability to form a colony.

The effect of isolated fungi as modulator of heat shock factor

Three fungal species [*Aspergillus niger* (F1), *Penicillium chrysogenum* (F2), *Aspergillus flavus* (F3)] and their combination (F1 with F2) were used as modulator of heat shock factors and biofertilizers to inoculate eight months old Mexican lime seedlings under heat stress (45±2 ºC for seven days) (Chhabra et al., 2009). The seedlings were planted and grown in sterile soil. Fungal inoculum preparation using autoclaved water in 1×10^6 CFU mi^{-1} colony formation (Fig. 1). The study was carried out in completely randomized design with 4 treatments, and three replications. The endophytes were inoculated three times (over three weeks).

After 120 days, morphological characteristics of the seedlings, including the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem

length, chlorophyll content (using SPAD-502; Konica Minolta, Osaka, Japan), trunk width, and numbers of leaves and branches were measured. The data were analyzed using a one-way analysis of variance (SAS Institute, 1988), and in the cases of significant differences, mean grouping was performed by the least significant difference (LSD) test ($P< 0.05$).

Results

Result of fungal isolation

From 1674 tissue segments belonging to 190 seaweed samples (79 red, 62 brown, and 49 green), in total, 566 colonies were recovered from red (285), brown (185), and green (96) seaweed species. The seaweeds collected from Bushehr province had the highest colonization rate (48.29%) (Table 3). In addition, the red seaweed species of Bushehr had the highest rate of colonization among three seaweed species both in summer (53.08%) and winter (50.61%) seasons. Likewise, red seaweed species, the brown seaweed species collected from Bushehr province, with 85 isolates, had a higher colonization rate than Hormozgan and Sistan and Baluchistan. Regarding green seaweed species, Hormozgan provinces with 43 isolates had a higher colonization rate than Bushehr and Sistan, and Baluchistan. Red seaweed species with 285 colonies had the highest isolation rate, followed by brown (185 colonies) and green (96 colonies) algae (Table 3).

Phylogenetic analyses

The phylogeny constructed using the sequence data of ITS1-5.8S-ITS4rRNA molecular region grouped all isolates in three distinct clusters (Fig. 2). Results of molecular identification, phylogenetic analysis, and GenBank accession numbers of all 20 sequences (Identified in the present study) and other supplementary information are shown in Table 4. Additional details regarding the endophytic fungi have been depicted in Table 5.

As descript in Table 5, all fungi isolation belonged to the Ascomycota phylum and had a transverse wall. Morphological and microscopy analysis showed that colonies' color varied from black to white (black, dark and light brown, gold, purple, dark and light green, yellow and white). Some fungi isolation like *Aspergillus niger* was mono-color (black), and some isolation like *A. carlsbadensis* was di color (white/purple). The spores' shapes were varied: Circle (in *Aspergillus*, *Penicillium* and *Paecilomyces* sp.), Spindle (in *Curvularia spicifera*) and Oval (in *Cladosporium macrocarpum*).

Spore array was in three forms, clustered (in *Aspergillus* and *Paecilomyces* sp. genus) (Fig. 3a), filamentous (in *Penicillium* genus) (Fig 3b), and sporadic (in *Curvularia spicifera* and *Cladosporium macrocarpum* genus) (Fig. 3c). All isolates have transverse walls in their septate hypha (Fig. 3d).

The fungi isolated by the F10 code (*Aspergillus carlsbadensis*) was extracted only from *G. folliifera* seaweed species collected from Bushehr province in the spring sea-

Table 3. Numbers of fungal colonies isolated from various seaweed samples collected from three province

Province	Season	No. of seaweed spices	Color	No. of segments	No. isolates generated	Colonization%
Hormozgan	Spring	18	5 red	45	14	31.11
			7 brown	63	9	14.28
			6 green	54	12	22.22
	Summer	21	8 red	72	12	16.66
			7 brown	63	16	25.39
			6 green	54	7	12.96
	Winter	37	15 red	135	46	34.07
			15 brown	135	31	22.96
			7 green	63	24	38.09
	Total	76	**76**	**684**	**171**	**25**
Sistan and Baluchistan	Spring	2	1 red	9	2	22.22
			1 green	9	1	11.11
	Summer	45	24 red	216	101	46.75
			6 brown	54	21	38.88
			15 green	135	13	9.62
	Winter	14	3 red	27	11	40.74
			7 brown	63	23	36.50
			4 green	36	10	27.77
	Total	61	**61**	**549**	**182**	**33.15**
Bushehr	Spring	11	5 red	45	15	33.33
			5 brown	45	23	51.11
			1 green	9	0	0
	Summer	17	9 red	81	43	53.08
			7 brown	63	25	39.68
			1 green	9	3	33.33
	Winter	25	9 red	81	41	50.61
			8 brown	54	37	68.51
			8 green	54	26	48.14
	Total	53	**53**	**441**	**213**	**48.29**
	Total+	190	**190**	**1674**	**566**	**33.81**

son (Fig. 4). It was individual isolation that did not found in any other seaweed species.

Salinity test

Although, most of fungal endophytes (85%) could grow properly on PDA medium incorporated with one molar NaCl concentration and got the number 8 and 7 ranking scores in salinity test, in 2 molar NaCl, only 60% of the isolates were able to grow decently. In 3 molar NaCl, only 35% of the isolates were grown (Table 6, Fig. 5 and Fig. 6).

Result of fungal endophytes inoculation as biofertilizer

After 120 days post-inoculation, Mexican lime seedlings were analyzed. As shown in Figure 8, fungal endophytes could improve the morphological characteristics of the Mexican lime seedlings. Selected fungal endophytes (F1, F2, F3, and F1F2),

Table 4. Name, identification code and accession number of the isolated endophytic fungi along with other additional information regarding their seaweed host, sampling site and sampling time

No	Fungi isolates	Code	Accession No.	Seaweed host	location	Season
1	*Aspergillus niger*	F1	MT420720	*Cladophoropsis* sp.	BU	Spring
2	*Penicillium chrysogenum*	F2	MT420723	*S. bov,* var. *attrimum*	BU	Spring
3	*A. flavus*	F3	MT420731	*Chaetomorpha* sp.	B. S	Summer
4	*P. chrysogenum*	F4	MT476156	*Gracilaria* sp.	RA	Summer
5	*Penicillium* sp.	F5	MT476164	*Champia parvulla*	BU	Spring
6	*A. terrus*	F7	MT476158	*Champia parvulla*	BU	Spring
7	*A. puniceus*	F8	MT476159	*Champia parvulla*	BU	Spring
8	*A. terrus*	F9	MT420850	*Hypnea* sp.	B. S	Summer
9	*A. carlsbadensis*	F10	MT476165	*G. follifera*	BU	Spring
10	*P. chrysogenum*	F11	MT476166	*S. angustifolium*	B. L	Summer
11	*A. terrus*	F14	MT420880	*Gracilaria* sp.	BE	Summer
12	*A. niger*	F15	MT420884	*Cladophoropsis* sp.	Q. I	Spring
13	*Curvularia spicifera*	F16	MT420887	*Iyengaria stellata*	B. L	Winter
14	*A. egyptiacus*	F17	MT420890	*Gracillaria* sp.	BU. P	Winter
15	*A. chevalieri*	F18	MT420889	*Gracilaria* sp.	Q. I	Winter
16	*P. chrysogenum*	F20	MT420891	*Gracilaria* sp.	B. A	Winter
17	*Cladosporium macrocarpum*	F24	MT420892	*S. boveanum*	B. A	Spring
18	*Paecilomyces* sp.	F25	MT476163	*Iyengaria stellata*	B. L	winter
19	*P. chrysogenum*	F29	MT420908	*G.persica*	B. A	Winter
20	*P. chrysogenum*	F33	MT476157	*Grateloupia lithophila*	B. A	Winter

BU, B. S, RA, B. L, BE, BU. P, Q. I, B. A denote Bushehr, Bandar-e Sirik, Ramin in Sistan and Baluchistan province, Bandar-e Lengeh, Beris in Sistan and Baluchistan province, Bushehr power plant, Qeshm Island and Bandar Abbas, respectively.

Table 5. Endophyte isolate code and Supplementary information of the endophytic fungi associated with some Iranian Macroalgae

Code	Taxa	Fugal order	phylum	Fungal class	Family
F1	*Aspergillus niger*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F2	*Penicillium chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F3	*A. flavus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F4	*P.chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F5	*Penicillum* sp.	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F7	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F8	*A. puniceus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F9	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F10	*A. carlsbadensis*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F11	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F14	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F15	*A. niger*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F16	*Curvularia spicifera*	Pleosporales	Ascomycota	Dothideomycetes	Pleosporaceae
F17	*A. egyptiacus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F18	*A. chevalieri*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F20	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F24	*Cladosporium macrocarpum*	Capnodiales	Ascomycota	Dothideomycetes	Cladosporiaceae
F25	*Paecilomyces* sp.	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F29	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F33	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae

were able to increase the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem length, trunk width, SPADE number, leaf number and branches number in the inoculated Mexican lime seedlings (Fig. 7). The combination of F1F2 isolates resulted in higher trunk width, leaf number, leaf fresh/dry weight, root fresh/dry weight, stem fresh weight, and root length compared to when they were used alone (Fig. 7). The highest chlorophyll SPAD and root width were obtained by F1 inoculation. The highest stem length was obtained by F3 inoculation (Fig. 7).

Significant differences were found in all morphological characteristics measured by colonizing the Mexican lime seedling with endophytic fungi (Table 7 and Fig. 8).

Discussion

Numerous research studies have shown that asymptomatic, systemic fungi that colonize the healthy leaves, stems, roots, reproductive organs of the host significantly affect the physiology, ecology, and reproductive biology (Bonnet et al., 2000; Clay

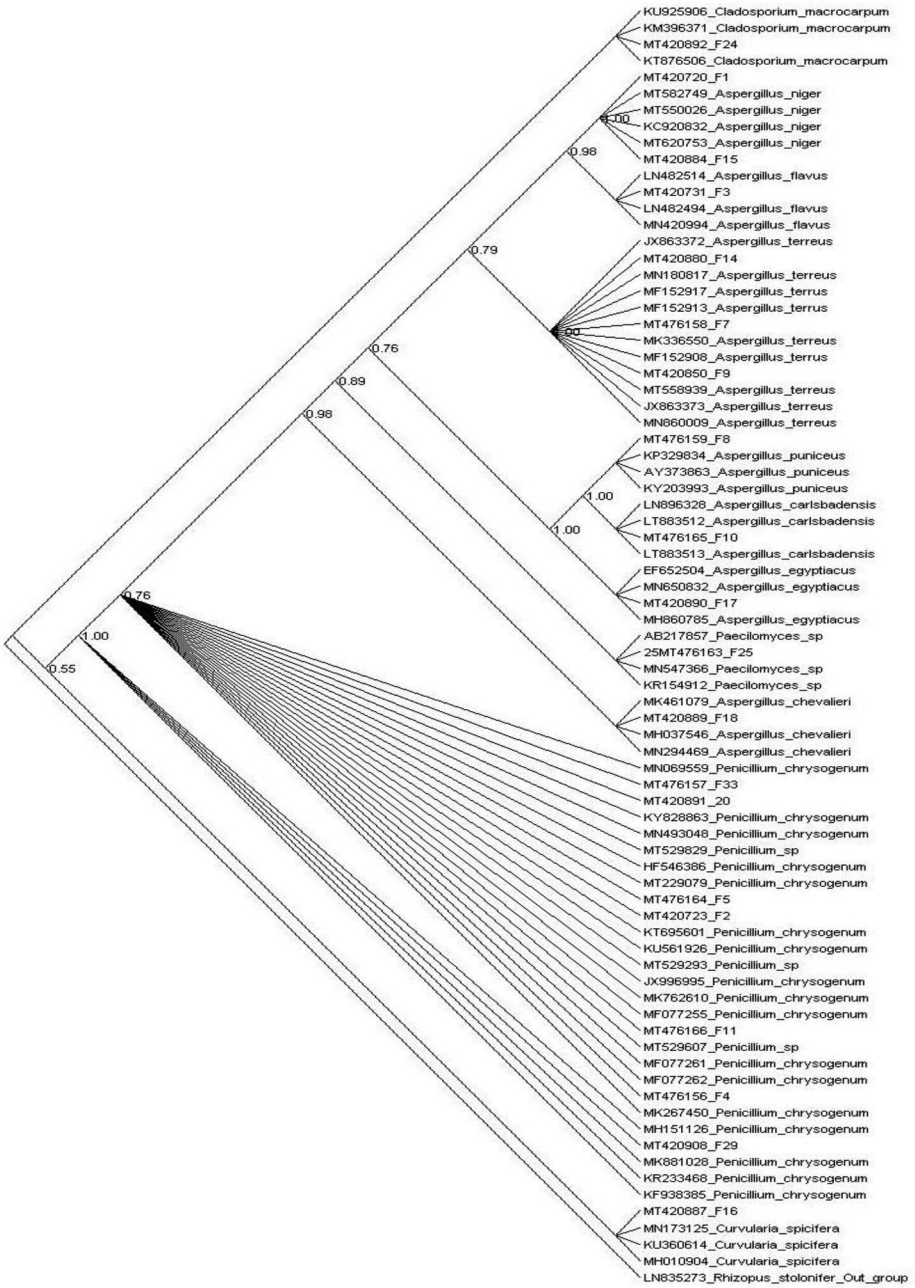

Fig. 2. The 50% majority rule consensus tree inferred from Bayesian analysis under the GTR + G + I model. The accession numbers of strains and their reference codes are shown

and Schardl, 2002; Clay et al., 2005; Malinowski and Belesky, 2006; Knop et al., 2007; Alfaro and Bayman, 2011) of the host plants. It showed that applying microbial products as inoculants can improve crop production under stress conditions or enhance disease resistance. Endophytic fungi can confer protection to hosts against insect pests and abiotic stresses (Thrower and Lewis, 1973; Clay and Schardl, 2002). The simulations of plant growth executed by plant growth promoters could be attributed to tolerance to biotic and abiotic stresses and improved plant nutrition (Machungo et al., 2009).

DNA analyzing method is a rapid approach

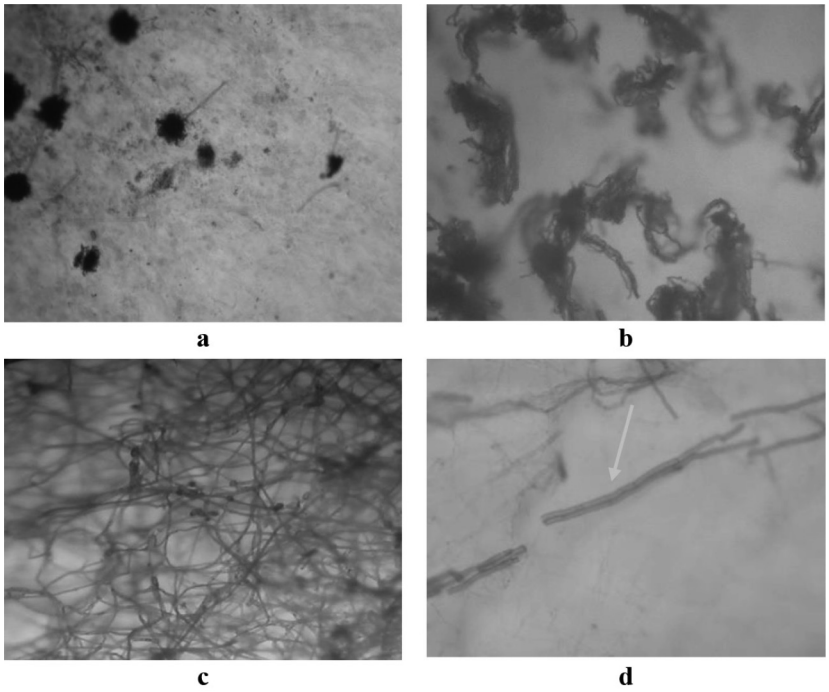

Fig. 3. Microscopic results for fungal isolates spore array; **a**: clustered form in *Aspergillus* genus, **b:** filamentous form in *Penicillium* genus and **c**: sporadic form in *Curvularia spicifera* genus, **d**. septate hypha in fungal isolates (a the isolates have transverse wall in their septate hypha).

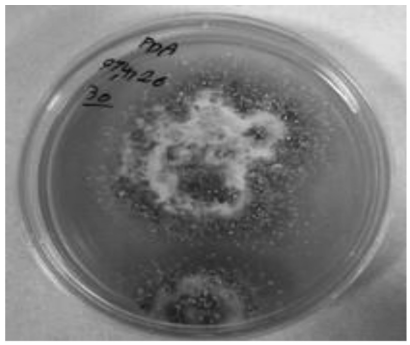

Fig. 4. *Aspergillus carlsbadensis* (F10), a fungal endophyte that was isolated just from *G. folliifera* species that collected from Bushehr Province in spring

to identifying fungi, especially in no sporulation endophytes (Lee et al., 2008; Zhang et al., 1996). The ITS1-5.8S-ITS4rRNA is a highly conserved region in fungi and can differentiate higher taxonomic levels. In contrast, ITS regions are highly variable and can be used to analyze lower taxonomic ones (Sugita and Nishikawa, 2003). Marine-derived endophytic fungi have been widely studied due to their bioactive metabolites (Bhadury et al., 2006; Newman et al., 2006). In the present study, the iden-

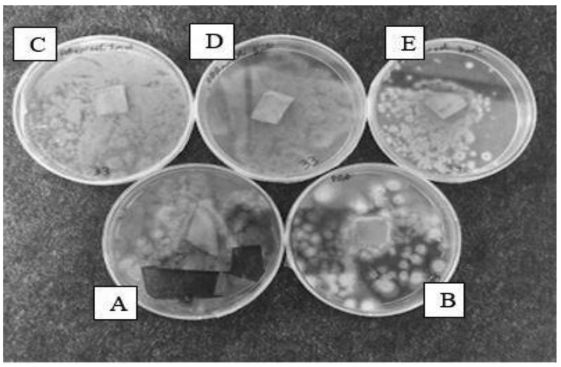

Fig. 5. Result of salinity test of the fungal endophyte (F33) on mediums incorporated with different NaCl concentrations (0, 1, 2, and 3 mol NaCl) after seven days; a, b, s, d, and e means: main stock, medium without NaCl, medium with 1 mol NaCl, medium with 2 mol NaCl and medium with 3 mol NaCl respectively

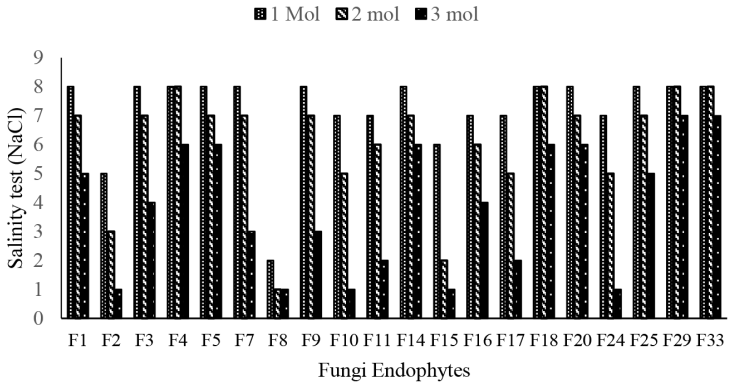

Fig. 6. Result of salinity test of the fungal endophytes on mediums incorporated with different NaCl concentrations (0, 1, 2 and 3 molar NaCl) after seven days

tification of fungi endophytes associated by different seaweed species (Rhodophyta, Chlorophyta, and Phaeophyta) collected from coastal regions of the Persian Gulf and Oman Sea was confirmed by PCR assay using universal primers (ITS1-5.8S-ITS4r-RNA sequence). Most isolates belonged to *Aspergillus* and *Penicillium* genera. The type of endophytes varied based on seaweed species, sampling site, and sampling season. All isolates belonged to the Ascomycota phylum (Table 5).

Phosphate is one of the essential compounds needed for plant growth, generally found in insoluble form and not utilized by plants. Plant growth-promoting fungi with phosphate solubilizing ability belong to *Aspergillus* and *Penicillium* genera (Noor-

Table 6. Physiological and morphological characteristics reports of endophytic fungi isolations

No	Code	1 mol	2 mol	3 mol	Colony color	Mono/Di color	Spore shape	Septate hypha
1	F1	8	7	5	Black	Mono	Circle/Clustered	+
2	F2	5	3	1	green	Mono	Circle/filamentous	+
3	F3	8	7	4	Green	Mono	Circle/Clustered	+
4	F4	8	8	6	Green	Mono	Circle/filamentous	+
5	F5	8	7	6	Green/creamy	Di	Circle/filamentous	+
6	F7	8	7	3	Brown/gold	Di	Circle/Clustered	+
7	F8	2	1	1	Brown	Mono	Circle/Clustered	+
8	F9	8	7	3	Brown	Mono	Circle/Clustered	+
9	F10	7	5	1	White/purple	Di	Circle/Clustered	+
10	F11	7	6	2	Black/white	Di	Circle/filamentous	+
11	F14	8	7	6	Yellow/green	Di	Circle/Clustered	+
12	F15	6	2	1	White	Mono	Circle/Clustered	+
13	F16	7	6	4	Dark gray	Mono	Spindle/sporadic	+
14	F17	7	5	2	Gray green	Di	Circle/Clustered	+
15	F18	8	8	6	Yellow	Mono	Circle/Clustered	+
16	F20	8	7	6	Purple	Mono	Circle/filamentous	+
17	F24	7	5	1	Dark green	Mono	Oval/ sporadic	+
18	F25	8	7	5	Dark green	Mono	Circle/Clustered	+
19	F29	8	8	7	Light brown	Mono	Circle/filamentous	+
20	F33	8	8	7	Dark green	Mono	Circle/filamentous	+

For salinity test, 1, 2, 3, 4, 5, 6, 7 and 8 means; very weak, weak, relatively weak, relatively intermediated, intermediated, relatively severe, severe and very severe, respectively.

jahan et al., 2019). Numerous studies indicate that *Aspergillus* and *Penicillium* are two common endophytes in different seaweed species (Suryanarayanan et al., 2010; Narayanan et al., 2013; Venkatachalam et al., 2015; Flewelling et al., 2015). Among the different genera identified in the seaweed species, the *Aspergillus* and *Penicillium* genus had the highest number of colonies (55% and 30%, respectively). Out of 190 seaweed samples, 32 isolates (16.84%) belonged to *A. niger*. This is the first study that describes indigenous fungal endophytes isolated from Iranian seaweeds. It has been revealed that most endophytic fungi isolated from plants are members of the Ascomycota or their anamorphs (Rungjindamai et al., 2008).

Seven different species of *Aspergillus* were identified in the present study, including *A. niger*, *A. terras*, *A. flavus*, *A. puniceus*, *A. carlsbadensis*, *A. egyptiacus* and *A. chevalieri*. We also found differences in endophyte colonization between different sam-

Table 7. Mean (± SE) of the studied attributes in the fungi colonized Mexican lime seedlings compared to non-inoculated seedlings

Characterizes	N	F1	IRCC	F2	IRCC	F1F2	IRCC	F3	IRCC
SPAD	37.56±0.04^{***b}	59.73±0.04^{***a}	59.02	59.66±0.03^{***a}	58.83	59.13±0.01^{***a}	57.42	59.53±0.04^{***a}	58.49
Trunk width	2.91±0.00^{***d}	3.93±0.00^{***b}	35.05	3.6±0.01^{***bc}	23.71	4.3±0.01^{***a}	47.76	3.37±0.01^{***c}	15.80
Leaf no	22.00±0.1^{***d}	30.00±0.27^{***c}	36.36	40.66±0.21^{***b}	84.81	66.33±0.08^{***a}	201.5	40.00±0.1^{***b}	81.81
Branches	2.66±0.03^{**b}	3.33±0.03^{**b}	25.18	3.66±0.03^{**b}	37.59	3.66±0.03^{**b}	37.59	5.33±0.03^{**a}	100.37
Stem length	15.33±0.03^{***d}	30.33±0.08^{***b}	97.84	25.66±0.11^{***c}	67.38	34.66±0.13^{***a}	126.09	38.00±0.1^{***a}	147.87
Leaf FW	4.99±0.02^{***c}	9.94±0.02^{***b}	99.19	10.06±0.01^{***b}	101.60	13.87±0.01^{***a}	177.95	10.02±0.09^{***b}	100.80
Leaf DW	0.45±0.00^{***c}	2.11±0.00^{***b}	368.88	2.10±0.00^{***b}	366.66	3.29±0.00^{***a}	631.11	2.16±0.01^{***b}	380
Root FW	5.03±0.02^{***d}	12.28±0.02^{***c}	144.13	16.66±0.01^{***b}	218.54	17.8±0.00^{***a}	253.87	15.99±0.04^{***b}	217.89
Root DW	1.36±0.02^{***d}	2.47±0.00^{***c}	81.61	3.19±0.00^{***b}	134.55	4.31±0.00^{***a}	216.91	3.44±0.01^{***b}	152.94
Stem FW	3.11±0.00^{***d}	6.06±0.02^{***c}	94.85	7.01±0.00^{***ab}	125.40	8.71±0.05^{***a}	180.06	7.81±0.01^{***ab}	151.12
Stem DW	1.53±0.00^{*b}	2.37±0.00^{*ab}	54.90	2.63±0.01^{*a}	71.89	2.59±0.06^{*a}	69.23	2.83±0.02^{*a}	84.96
Root length	29.5±0.16^{***c}	32.16±0.05^{***c}	9.01	35.83±0.08^{***b}	21.45	44.73±0.1^{***a}	51.62	44.16±0.04^{***a}	49.69
Root width	27.00±0.1^{***b}	55.5±0.16^{***a}	105.55	55.33±0.15^{***a}	104.92	51.5±0.07^{***a}	90.74	52.16±0.06^{***a}	93.18

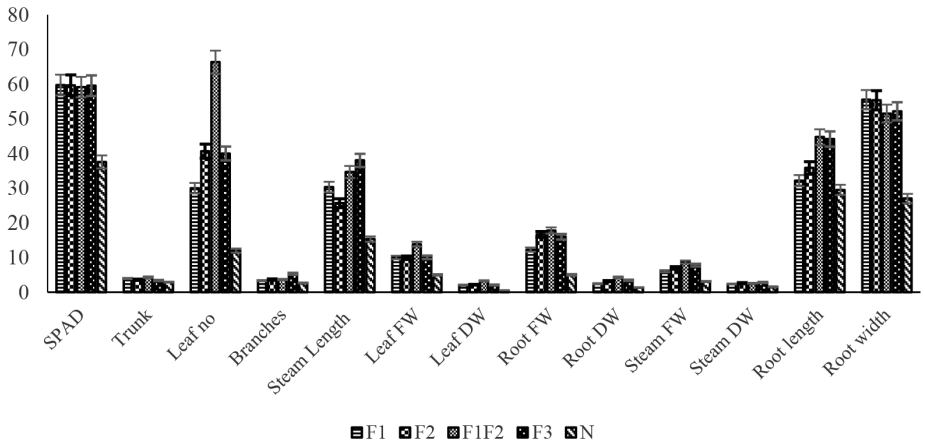

Fig. 7. Morphological analysis (Chlorophyll SPAD number, trunk width, leaf and branch number, stem length, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, root length and root width) of Mexican lime seedling after 120 days. N, means; without inoculation. F2 is the F4 isolate in Table 3.

Fig. 8. Mexican lime seedling inoculated with fungal endophytes (F1+F2) compared to uninoculated seedling (N), 120 days post inoculation and seven days heat stress (45±2 ℃)

pling sites. High differences in the number of colonization were found between the three provinces in which the sampling was made. The highest number of isolates were found in the seaweeds collected from Bushehr province (213 isolates), followed by Sistan and Baluchistan (182 isolates) and Hormozgan (171 isolates) provinces. Also, Bushehr had the highest colonization rate (48.29%), followed by Sistan and Bal-

uchistan (33.15%) and Hormozgan (25%). These results could be due to Bushehr's position in the Persian Gulf region.

In this study, it was found that some endophytic fungi were restricted only to one seaweed species. For instance, some fungal endophytes such as *A. carlsbadensis* was observed only in *G. folliifera* collected from Bushehr in the spring season. The ecological and environmental conditions such as seawater temperature or salinity rate may affect the colonization of host tissues by endophytes. In another example, the species *G. persica* collected from Qheshm Island in spring had no endophytes. When it was collected from Bandar Abbas in winter, it was associated with four fungal endophytes.

In the genus *Gracilaria*, the highest number of isolates was observed in summer (66 isolates) in Ramin (38 isolates) and Beris (13 isolates) regions. The association of these seaweed species with high salinity-resistant endophytes may be one of the reasons for their abundance in the sea. Interestingly, some seaweeds (*Gracilariopsis persica*) had no endophyte in warm seasons whereas, in cool winter, several fungal endophytes were isolated from them. *Gracilariopsis persica* is an endangered species that has limited growth in warm seasons. The association of endophytes with this species in cold seasons seems to be an adopted defense mechanism to save this species from extinction.

In the current study, the ability of endophytic fungi to produce colony on the PDA medium incorporated with different concentrations of NaCl was studied and found, in one mol NaCl; most of the isolates (85%) were able to colonize the medium and allocated the score of 7 and 8 in the test of resistance to salinity conditions. In 2 mol NaCl, 60% of the studied isolates could produce a colony. The species *Penicillium* chrysoganum (F4), *A. chevalieri* (F18), *P. chrysogenum* (F29), and *P. chrysogenum* (F33) were able to colonize in the PDA culture over the experiment fully. These fungal endophytes were isolated from *Gracilaria* sp., *Gracilariopsis persica*, and *Grateloupia lithophila,* indicating the resistance to salinity in endophytes can vary based on seaweed species (Koch et al., 2017). In addition, it seems that the genus *Penicillium* was more tolerable to salinity than the other studied genus (Yadav et al., 2018).

Identifying the relationship between seaweeds and their associated endophytes provides important opportunities for research. Some endophytes like *Aspergillus* and *Penicillium* genus have shown potential to produce pectinases, cellulases, xylanases, and proteases (Bezerra et al., 2012). Over 300 natural endophyte-derived products were identified from 32 endophytic fungi, with 22% of the investigated fungi being from the *Aspergillus* genus (Flewelling et al., 2015).

Aspergillus niger (MT420720), *P. chrysogenum* (MT476156), and *A. flavus* (MT420731) for F1, F2, and F3 isolates, respectively, were used for inoculation purposes. We selected these isolates due to their high frequency in the studied seaweed

species in the current study.

We showed that the fungal endophytes F1, F2, F3, and F1F2 could improve the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem length, trunk width, SPAD number, the number of leaf and branches in inoculating Mexican lime seedlings. Therefore, the fungal community present in the internal parts of the roots and foliage of Mexican lime seedlings has the potential role of promoting plant growth. One of the problems of lemon trees in summer is susceptibility to the fungus *Nattrassia*. In the present study, the endophyte-inoculated seedlings did not have any exposure to *Nattrassia* and had a higher growth rate than the control. Ngamau et al. (2014) showed that endophytes, through P solubilization or siderophore production (iron chelation), can help plants grow better. For instance, Nadeem et al. (2010) studied the plant growth-promoting activity and stress resistance capability of the genus *Penicillium* and *Aspergillus*. They found these two endophytic fungi produced physiologically active gibberellins. A previous study showed that seaweeds with high endophyte colonization had more ability to metal (Fe, Cu, Zn) absorption (Baghazadeh et al., 2020). So, some endophytes have a high capacity to metal absorbance from soil to host plants. It needs more investigation to understand the skills of the other seaweeds fungi endophytes obtained in this study, especially for biofertilizer aims.

Overall, our findings indicate that the fungal endophyte diversity in the seaweeds can be affected highly by seaweed species, sampling sites, and sampling season. Although this is the first study on fungal endophytes of Iranian seaweed, more research is needed to identify the functional and ecological significance of these fungal endophytes. This study showed that some of the identified species have a high potential to increase plant tolerance to salinity conditions that can be used to produce bio-fertilizers in the future. Accordingly, to reach this critical goal, establishing microbial reservoirs of seaweed endophytes is highly recommended.

Acknowledgments

The authors wish to thank Dr. Reza Rabiei, the head of the Natural Resources Department of Agriculture and Natural Resources Researches and Education Center of Hormozgan, for providing the facilities and their technicians' assistance for periodical algal samples collection. Also, we appreciate all members of the Biotechnology laboratory of Hormozgan University and the Environmental protection Department of Hormozgan for their kind collaboration in all processes of the experiments of the current study.

References

Alfaro AP and Bayman P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. Annual Review of Phytopathology. 49:291–315.

Baghazadeh Daryaii L, Samsampour D, Bagheri A, Sohrabipou J. (2020). High content of heavy metals in seaweed species: A case study in the Persian Gulf and the Gulf of Oman in the southern coast of Iran. Journal of Phycological Research. 4 (2): 544-560.

Bezerra JDP, Santos MGS, Svedese VM. (2012). Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production. World Journal of Microbiology and Biotechnology. 28 (5):1989-1995.

Bhadury P, Mohammad BT, Wright PC. (2006). The current status of natural products from marine fungi and their potential as anti-infective agents. Journal of Industrial Microbiology and Biotechnology. 33:325-337

Bonnet M, Camares O, Veisseire P. (2000). Effects of zinc and influence of Acremonium lolii on growth parameters, chlorophyll-a fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne* L. cv Apollo). Journal of Experimental Botany. 51 (346): 945-953.

Chhabra ML, Dhawan A, Sangwan N, Dhawan K, Singh D. (2009). Phytohormones induced amelioration of high temperature stress in *Brassica juncea* (L.) Czern and Coss. Proceedings of 16th Australian Research Assembly on Brassicas, Ballarat, Australia. 9-11.

Clay K and Schardl C. (2002). Evolution origin and ecological consequences of endophyte symbiosis with grasses. American Naturalist. 160:99-127.

Clay K, Holah J, Rudgers JA. (2005). Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. Proceeding of the National Academy Sciences of the United States of America. 102: 12465-12470.

Davati N, Najafi MBH. (2013). Overproduction strategies for microbial secondary metabolites: A review. International Journal of Life Sciences and Pharma Research. 3: 23-27.

Flewelling AJ, Currier J, Gray C, Johnson A. (2015). Endophytes from marine macroalgae: promising sources of novel natural products. Current Science. 109 (1): 88-111. https://www.jstor.org/stable/24905694.

Jimenez C. (2018). Marine natural products in medicinal chemistry. ACC Medicinal Chemistry Letters. 9: 959-961. DOI: 10.1021/acsmedchemlett.8b00368

Kjer J, Debbab A, Aly AH, Proksch P. (2010). Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nature Protocols 5 (3):479-490

Knop M, Pacyna S, Voloshchuk N, Kjant S, Mullenborn C, Steiner U, Kirchmair M, Scherer HW, Schulz M. (2007). *Zea Mays*: Benzoxalinone Detoxification under sulfur deficiency conditions-A complex allelopathic alliance including endophytic *Fusarium verticilloides*. Journal of Chemistry Ecology. 33 (2): 225-237.

Koch ED, Honig J, Vaiciunas J, Meyer WA, Bonos SA. (2017). Effect of endophyte on salinity tolerance in *Perennial ryegrass*. International Turfgrass Society Research Journal. 13 (1):459-465.

Kusari S and Spiteller M. (2012). Metabolomics of endophytic fungi producing associat-

ed plant secondary metabolites: progress, challenges and opportunities. In: Roessner U (ed) Metabolomics. Rijeka, Croatia, In-Tech. 241-266. Doi: 10.5772/31596.

Lee JS, Lee HK, Hur JS, Andreev M, Hong SG. (2008). Diversity of the lichenized fungi in King George Island, Antarctica, revealed by phylogenetic analysis of partial large subunit rDNA sequences. Journal of Microbiology and Biotechnology. 18:1016-1023.

Machungo C, Losenge T, Kahangi E, Coyne D, Dubois T, Kimenju J. (2009). Effect of endophytic *Fusarium oxysporum* on growth of tissue-cultured Banana plants. African Journal of Horticultural Science. 2:160-167.

Malinowski DP and Belesky DP. (2006). Ecological importance of Neotyphodium sp. grass endophyte in agroecosystems. Grassland Science. 52 (1): 23-28.

Mejía LC, Herre EA, Sparks JP, Winter K, Garcia MN, Van Bael SA, Stitt J, Shi Z, Zhang Y, Gultinan MJ, Maximova SN. (2014). Pervasive effects of a dominant foliar endophytic fungus on host genetic and phenotypic expression in a tropical tree. Frontiers in Microbiology. Doi:10.3389/fmicb.2014.00479

Miliute I, Buzaite O, Baniulis D, Stanys V. (2015). Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. Zemdirbyste-Agriculture. 102 (4): 465-478.

Nadeem A, Hamayun M, Khan SA, Khan AL, Lee IJ, Shin DH. (2010). Gibberellin-producing endophytic fungi isolated from *Monochoria vaginalis*. Journal of Microbiology and Biotechnology. 20 (12): 1744-1749.

Narayanan K, Chopade N, Vasanth Raj P, Subrahmanyam VM, Venkata Rao J. (2013). Fungal chitinase production and its application in biowaste management. Journal of Scientific and Industrial Research. 72: 393-399.

Newman DJ and Hill RT. (2006). New drugs from marine microbes: the tide is turning. J. Ind. Microbial Biotechnology. 33: 539-544.

Ngamau CN, Matiru VN, Tani A, Muthuri CW. (2014). Potential use of endophytic bacteria as biofertilizer for *sustainable banana* (Musa spp.) production. African Journal of Horticultural Science. 8:1-11.

Noorjahan A, Aiyamperumal B, Anantharaman P. (2019). Isolation and Charecterisation of Seaweed Endophytic Fungi as an Efficient Phosphate Solubiizers. Biosciences Biotechnology Research Asia. 16 (1):33-39.

Rateb ME and Ebel R. (2011). Secondary metabolites of fungi from marine habitats. Natural Product Reports. 28 (2):290-344.

Rodriguez RJ, White Jr JF, Arnold AE, Redman ARA. (2009). Fungal endophytes: diversity and functional roles. New Phytologist. 182 (2): 314-330.

Ronquist F and Huelsenbeck JP. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19 (12): 1572-1574.Doi:10.1093/bioinformatics/btg180.

Rungjindamai N, Pinruan U, Choeyklin R, Hattori T, Jones EBG. (2008). Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oilpalm, *Elaeis guineensis*, in Thailand. Fungal Diversity. 33: 139-161.

SAS Institute. (1988). SAS/StAT User Guide, Release 6, 06, SAS Inst., Cary, NC.

Sohrabipour J and Rabiei R. (2007). The checklist of green algae of the Iranian coast lines of the Persian Gulf and Gulf of Oman. Iranian Journal of Botany. 23 (1):146-149.

Sohrabipour J and Rabiei R. (2008). Rhodophyta of Oman Gulf (South east of Iran). The Iranian journal of botany. 14 (1): 70-74.

Soltani J and Moghaddam MSH. (2015). Fungal endophyte diversity and bioactivity in the mediterranean cypress *Cupressus sempervirens*. Current Microbiology. 70 (4):580-586.

Strobel G, Daisy B. (2003). Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews. 67 (4):491-502.

Subramani R, Aalbersberg W. (2013). Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery. Applied Microbiology and Biotechnology. 97 (21): 9291-9321.

Sugita T and Nishikawa A. (2003). Fungal identification method based on DNA sequence analysis: reassessment of the methods of the pharmaceutical society of Japan and the Japanese pharmacopoeia. Journal of Health Science. 49 (6): 531-533.

Suriya J, Bharathiraja S, Krishnan M, Manivasagan P, Kim SK. (2016). Extremozymes from Marine Actinobacteria. Advances in Food and Nutrition Research. 79: 43-66.

Suryanarayanan TS. (1992). Light-incubation: a neglected procedure in mycology. Mycologist 6:144

Suryanarayanan TS, Venkatachalam A, Thirunavukkarasu N, Ravishankar JP, Doble M, Geetha V. (2010). Internal mycobiota of marine macroalgae from the Tamilnadu coast: distribution, diversity and biotechnological potential. Botanica Marina. 53: 457-468.

Suryanarayanan TS. (2013). Endophyte research: going beyond isolation and metabolite documentation. Fungal Ecology. 6: 561-568.

Thrower LB, Lewis DH. (1973). Uptake of sugars by *Epichloe typhina* (Pers. Ex Fr.) Tul, in culture and from its host, *Agrostis stolonifer* L. New Phytologist. 72: 501-508.

Vatsyayan N, Ghosh AK. (2013). Isolation and characterization of microbes with biofertilizer potential. IOSR Journal of Environmental Science, Toxicology and Food Technology. 7 (4):5-9.

Venkatachalam A, Guvinda RMB, Thirunavukkarasu N, Suryanarayanan TS. (2015). Endophytic fungi of marine algae and seagrasses: a novel source of chitin modifying enzymes. Mycosphere. 6(3):345-355. Doi:10.5943/mycosphere/6/3/10.

White Jr JF, Morrow AC, Morgan-Jones G, Chambless DA. (1991). Endophyte-host associations in forage grasses. XIV. Primary stromata formation and seed transmission in *Epichloë typhina*: developmental and regulatory aspects. Mycologia. 83 (1):72-81.

Yadav AN, Verma P, Kumar V, Sangwan P, Mishra S, Panjiar N, Gupta VK, Saxena AK. (2018). Biodiversity of the genus *Penicillium* in different habitats. New and Future Developments in Microbial Biotechnology and Bioengineering. 3-18.

Yuan ZL, Zhang CL, Lin FC, Kubicek CP. (2010). Identity, diversity, and molecular phylogeny of the endophytic mycobiota in the roots of rare wild rice (*Oryza granulate*) from a nature reserve in Yunnan, China. Applied and Environmental Microbiology.76 (5): 1642-1652.

Zhang D, Yang Y, Castlebury LA, Cerniglia CE. (1996). A method for the large-scaleisolation of high transformation efficiency fungal genomic DNA. FEMS Microbiology Letters. 145 (2): 261-265.

Zhang Y, Mu J, Feng Y, Kang Y, Zhang J, Gu P, Wang Y, Ma L, Zhu YH. (2009). *Broadspectrum* antimicrobial epiphytic and endophytic fungi from marine organisms: Isolation, bioassay and taxonomy. Marine Drugs 7 (2): 97-112. Doi:10.3390/md7020097.

Zhang P, Li X, Wang BG. (2016). Secondary metabolites from the marine algal-derived endophytic fungi: Chemical diversity and biological activity. Planta Medica. 82 (09/10): 832-842.

Molecular Identification of *Caulerpa selago* as a New Record For The Persian Gulf

Mojib Salehi balashahri[1*], Jelveh Sohrabipour[2], Mohammad Mehdi Dehshiri[3], Iraj Mehregan[4]

Received: 2021-08-28 Revised and accepted: 2021-10-22

Abstract

Caulerpa is a genus of siphonous green algae belonging to Caulerpaceae which consists of about 103 accepted species of tropical to subtropical seas. Recently, some species of the genus have expanded their distribution into more temperate environments such as the Mediterranean Sea. Due to their bioactive compound and secondary metabolites, content species of this genus have many medicinal uses in various countries, especially in some countries such as the Philippines, Malaysia, and Vietnam, which are used as vegetables. The extract of this alga has antibacterial, anti-viral, and anti-inflammatory properties. Due to this economic and scientific importance, accurate identification of these species is very important. Morphological similarities between some species of this genus and phenotypic changes caused by ecological factors have caused some complications and problems with inaccurate identification at the species level. For this reason, in addition to morphological features, phylogenetic analyzes are also used to accurately identification of the members of this genus. In this study, a molecular phylogeny was inferred from chloroplast *tuf*A sequences of 35 taxa, which of them a sequence of *Caulerpella ambigua* used as an outgroup. The results confirmed the presence of *Caulerpa selago* in the algal flora of Iran which is the first record of this species from the coastlines of the Persian Gulf and Gulf of Oman in the south of Iran.

Keywords: *Caulerpa selago*, Persian Gulf, Gulf of Oman, *tuf*A gene.

Introduction

Caulerpa is a genus of seaweed in Caulerpaceae (green algae). This family belongs to the Bryopsidophyceae (Van den Hoek et al., 1995), a class consisting of algae with the coenocyticthallus. Their thallus is composed essentially of a single cell that develops into a system of branching siphons, stolons, and erect branches. The family Caulerpaceae includes two genera,

1- Islamic Azad University, Science and Research Branch , Tehran, Iran
2- Agriculture and Natural Resources Research and Education center of Hormozgan, Agriculture Research, Education and Extension Organization (AREEO)
3- Department of Biology, Borujerd Branch, Islamic Azad University, Borujerd, Iran
4- Department of Biology, Science and Research Branch , Islamic Azad University, Tehran, Iran
*Email address: m.salehibalash@gmail.com

Caulerpa J.V. Lamouroux (a species-rich genus) and the monotypic genus *Caulerpella* Prudhomme and Lokhorst (Draisma et al., 2014). Thalli of the genus *Caulerpa* are composed of a horizontal rhizome (stolon), downward branched rhizoids, and upright branches (assimilators) that bear distinctive branchlets (ramuli), which the characteristics are mainly used in species identification (Fritsch, 1965). More than 350 species and infraspecific taxa globally are reported as members of the genus *Caulerpa* (Belton et al., 2014), of which 103 taxa have been verified as accepted taxon (Guiry and Guiry, 2021). Species of the genus are characterized by differences in branching patterns of erect fronds, forms and arrangement of branchlets (ramuli), and stolen forms. These different morphological characters are commonly used for species delimitation conditions which cause phenotypic plasticity in all these characters, also leads to much confusion, and results in a large number of synonyms, including subspecies, varieties, forms, and "ecads" (Coppejans and Prud'homme van Reine, 1992; Belton et al., 2014). This Phenotypic plasticity is the source of taxonomic difficulty in many algal groups, sometimes leading to misidentifications that cause incorrect results in biodiversity estimation and confusing classification (Belton et al., 2014). Molecular study as a modern tool helps phycologists delimit this problem and achieve a convenient category in taxonomically challenging groups (Saunders and Kucera, 2010; Belton et al., 2014). Some molecular studies (Sauvage et al., 2013; Belton et al., 2014) showed the need for taxonomic revision in the *Caulerpa* genus.

Some *Caulerpa* species, such as *Caulerpa cylindracea* and *C. taxifiolia*, may cause severe environmental damage as invasive species (Preez et al., 2020). *C. lentillifera* (sea grapes) commercially has grown in ponds and consumed in the Philippines, Indonesia, and Vietnam. The species is rich in polyunsaturated fatty acids (PUFAs) (Saito et al., 2010), multiple essential amino acids, minerals, dietary fibers, vitamin A and Vitamin C (Matanjun et al., 2009), and has low levels of lipids (Niwano et al., 2009). The species can have improved metabolism of glucose, reduced inflammation, decreased bodyweight, reduced LDL, and increased HDL-cholesterol concentrations in high-fat diet-fed rats (Matanjun et al., 2009, 2010). In addition, *Caulerpa* species have some secondary metabolites such as flavonoids, Caulerpin, caulerpenyne (Meyer and Paul, 1992), and siphonaxanthin, which is a carotenoid found in green algae (Zheng et al., 2018). The antidiabetic effect of *C. lentillifera* has also been shown in a study by Khairuddin et al. (2020). Some species of this genus, such as *C. racemosa* used in manufacturing lotions due to the high level of antioxidants compounds that are good for the skin (Susilowati et al., 2019).

Diini (2013) has stated that one marine resource that has the potency to develop as functional foods is *C. racemosa*.

Some species can be aggressive from time

to time, such as *C. taxifolia* and *C. cylindracea*, which cause many problems in the Mediterranean Sea (Najdek et al., 2020a; 2020b; Chen et al., 2019). Due to this economic and scientific importance, accurate identification of the members of this genus is essential.

For the first time, a species of *Caulerpa*, *C. sertularoides*, and one new variety, *C. sertulariodes* var. *farlowii*, were reported from the Persian Gulf (Nizamuddin and Gessner, 1970). Sohrabipour and Rabiei (1996) reported 16 new records of marine algae, of which seven species, including *C. peltata* and *C. racemosa* were the new records for the Persian Gulf. Also, Sohrabipour and Rabiei (1999a, b) published a list of seaweeds from the shores of the Persian Gulf and the Gulf of Oman, in which 153 species of algae (including seven species of *Caulerpa*) were reported. In 2007, Sohrabipour and Rabiei presented a checklist of 62 species of green seaweeds from Iranian seashores of the Persian Gulf and Gulf of Oman. This checklist reported 10 species of the genus *Caulerpa*, including *C. fastigiata*, *C. manorensis*, *C. Mexicana*, *C. peltata*, *C. racemosa* var. *macrophysa*, *C. sertulariodes* f. *farlowii*, *C. sertularioides* f. *sertulariodes*, *C. taxifolia*, *C. brachypus* and *C. scalpellformis*. Some other studies on marine algae on the Iranian coast of the Persian Gulf and the Gulf of Oman have mentioned some species of *Caulerpa* (Gharanjik, 2000; RohaniGhadekolaei et al., 2007; Shams and GhaedAmini (2017).

Some *Caulerpa* species also have been reported from Arabian countries at southern coastlines of the Persian Gulf (Newton, 1955; Basson, 1979a; 1979b; 1992; Mshigeni and Dorgham, 1987; Dorgham 1990; De Clerck and Coppejans,1994; 1996; Rizk et al., 1999; Al-Abdessalaam, 2007; John, 2005; 2012).

In the current study, we used a combination of morphology and molecular studies because of some complications in identifying the genus *Caulerpa* on the Iranian shores of the Persian Gulf and the Gulf of Oman, to make clear the species boundary of this genus in the area.

Material and methods

Sample collection

Samples of the *Caulerpa* genus were collected from different intertidal areas of the Persian Gulf and the Gulf of Oman on the southern coastlines of Iran. After cleaning all debris, the samples were washed with sterile seawater and dried in a paper towel, and then dried on herbarium sheets; a part of each sample was dried in plastic bags containing silica gel and stored at -20 °C. Voucher specimens were deposited algal herbarium of Agriculture and Natural Resources Research and Education Center of HormozganProvince. Localities of collected samples are presented in Table 1.

Molecular studies

The silica gel dried samples were used for DNA extraction. The dried pieces of each specimen were subjected to total genomic DNA extraction using the Olsen CTAB modification method (Promega, Madison,

Table 1. Localities of collected samples at southern coastlines of Iran

Location	Station name	Longitude	Latitude
Jask	Jask coastal cape	57.764019	25.636320
	Saheli park	57.777129	25.640710
	Fajr seashore	56.232606	26.981241
Qeshm	Do koohak	56210783	26.996001
	Governorship beach	56.264558	26.969403
	Govatr port	61.501707	25.165417
Chabahar	Ramin	60.735824	25.272164

WI, USA) or Qiagen Kit. The extracted DNA was used for PCR amplification of the chloroplastic gene, *tuf*A, using the primers of stuff 5-TGAAACAGAAMAWCGTCATTATGC-3 as forward and *tuf*AR 5-CCTTCNCGAATMGCRAAWCGC-3 as revers (Fama et al., 2002). Polymerase chain reaction (PCR) was performed using a Multigene Thermal cycler TC9600-G (Lab Net International Inc, Edison, NJ, USA). The PCR reactions were performed using a PCR master mix of 13 µl consisting of 2.5 mM $MgCl_2$, 0.5 mm each primer, 0.2 mM each dNTP, 1.0 M Betaine, 0.5 units of Taq DNA polymerase purchased from Pishgam Biotech Company, Tehran, Iran (http://www.pishgam.com) and 0.5-1.0 µl of template DNA. The PCR program started with an initial denaturation at 96 °C for 5 minutes, followed by 40 cycles of denaturation (96 °C for 50 s.), primer annealing (48 °C for 1 min), and extension (72 °C for 2 min), followed by a final extension step at 72 °C for 4 min. PCR products were sent to Pishgam BiotechCompany (http://www.pishgam.com) for sequencing using the primers of the initial PCR reactions.

The obtained raw DNA sequences were edited using ChromasPro ver. 1.5 (Technelysium Pty Ltd, Queensland, Australia). The edited sequences were blasted in NCBI, and the sequences with higher similarities were accrued. The 35 sequences, including two sequences obtained current study and two sequences of *Caulerpella ambigua* (as outgroups), were aligned manually using BioEdit v.7.0.9.0 (Hall, 1999). They were rearranged with the Clustal X n.2.0.8 (Larkin et al., 2007). The best-fitted model for (1) maximum likelihood (ML) and (2) Bayesian (BI) analysis were selected using the results of KAKUSAN version3 (Tanabe, 2007) analyses. In the BI analyses, the Markov chain Monte Carlo method was used for 2000000 generations, and data sampling was done every 100 generations. The likelihood scores stabilized after 200,000 generations, and a 'burn-in' of 400,000 generations was used. To assess the level of variation in the provided sequences, the PAUP 4.0b.10 (Swofford, 2002) used to determine absolute distance and uncorrected pairwise genetic distances, excluding gaps and ambiguities. PAUP

version 4.0b.10 (Swofford, 2002) were used for maximum parsimony (MP) analyses which constructed trees using a heuristic search algorithm with 1000 random sequence additions, TBR branch swapping, using unordered and unweighted characters. The bootstrap values were generated using 1000 replicates.

Results

Morphological studies

Caulerpa selago (Turner) C. Agardh, 1817

Description: Thallus make a mat of dark to grass green in color on sandy or rocky substrates (Fig. 1A), consisting of creeping (true stolon with the rhizophorous branches) and erect axes Figure 1(B and C). entirely covered with upwardly curved branchlets (ramuli) (Fig. 1D) at the lower parts of some erect axes, the branchlets collapsed and were conspicuously roughened by the stub bases of the discarded ramuli. Stolons dark green to pale in color, cylindrical, prostrate, creeping, and irregularly branched; erect axes are primarily simple, covered with closely imbricated, cylindrical, and acute ramuli. Vertical axes up to 30 cm high and prostrate stolons up to 2 mm diameter and can grow more than 1 m, ramuli simple, cylindrical, multi-seriate, alternate to irregularly arranged, upwardly curved, with mucronate apex, shrinking and wrinkled when dry and do not adhere to the herbarium sheets.

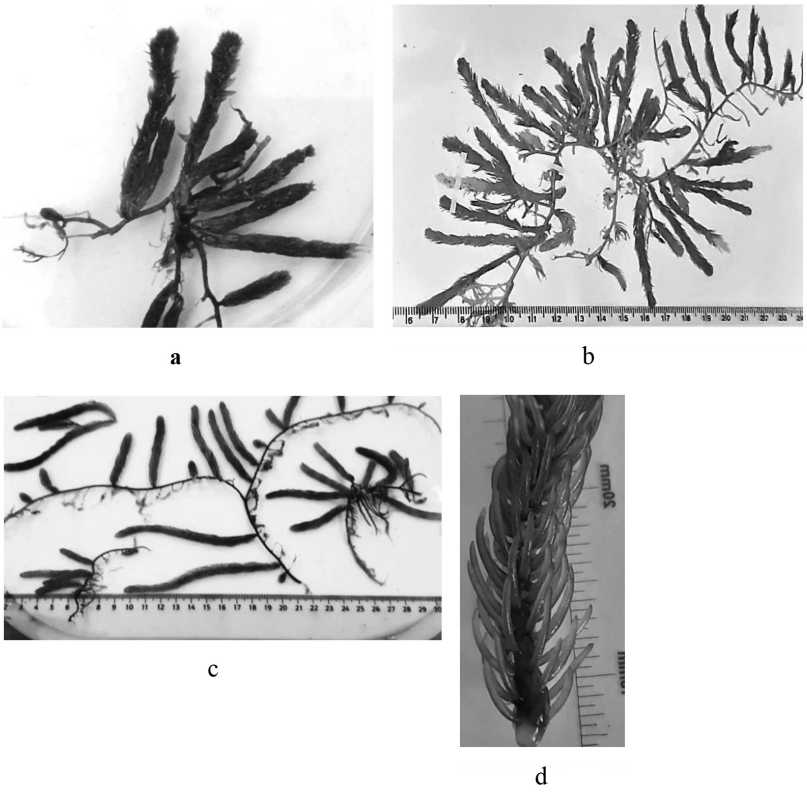

Fig. 1. *Caulerpa selago* collected from southern coastlines of Iran: (A) a mat of the species on sandy bottom, (B) dried herbarium specimens, (C) fresh samples of the specimens shows the creeping and erect branches and rhizoids (D) upwardly curved and mucronate form and size of ramulies on erect branches.

Molecular studies

The set of the sequences alignment contained 760 base pairs (bp), of which 578 characters are constant, 23 variable characters are parsimony-uninformative, and several parsimony-informative is 159. The aligned set consisted of 37 *tuf*A sequences, including two new sequences generated in the current study and two sequences of *Caulerpella ambigua* as outgroups were subjected to three methods of phylogenetic analyzes. The results of the phylogenetic analyses based on all three ways revealed that the two new generated sequences from Iranian coastlines of the Persian Gulf were clustered with a full bootstrap value in a clade, including a unique sequence from the samples of *C. selago*, which was submitted in GenBank from the Egyptian coast of the Red Sea (Fig. 2). This clade has a sister relationship with a branch including *C. amanuensis* and *C. cylindracea*, which biogeographically have distributed in subtropical and tropical areas.

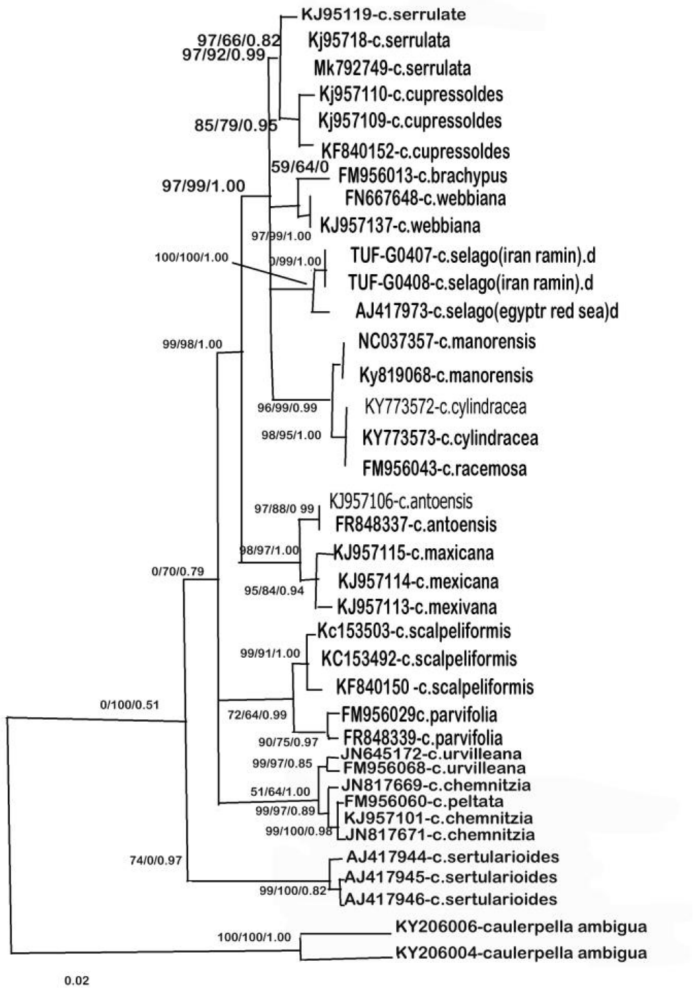

Fig. 2. The constructed ML tree based on 38 *tuf*A sequences. Bootstraps and posterior probability support for each node are arranged as follow ML/MP/BI. The taxa in bold show the obtained sequences from Iranian specimens and *Caulerpa selago* from Red Sea.

Discussion

In this study, we aimed to do an accurate taxonomic analysis of *Caulerpa* members in the marine waters of the Persian Gulf and the Gulf of Oman on the southern coastlines of Iran. A part of the study focused on *Caulerpa* selago was presented in the current report. In the previous studies ten species including *C. fastigiata*, *C. manorensis*, *C. mexicana*, *C. peltata*, *C. racemosa* var. *macrophysa*, *C. sertulariodes* f. *farlowii*, *C. sertularioides* f. *sertulariodes*, *C. taxifolia*, *C. brachypus* and *C. scalpellformis* have been reported from the area (Nizamuddin and Gessner, 1970; Sohrabipour and Rabiei, 1996; 1999a; 1999b; 2007; Sohrabipour et al., 2004; Kokabi and Yusefzadi, 2015).

In the images provided by Sohrabipour and Rabiei (1996, 1999a, 1999b), it seemed that the specimens of *C. selago* were reported as *C. sertularioides* f. *farlowii* (Weber Bosse) Børgesen (1907) based on Nizamuddin and Gessner (1970) report from Abu-Dabi, United Arab Emirate. These reports were repeated in published papers and checklists from the area (Sohrabipour and Rabiei, 2007; John and Al-Thani, 2014; Kokabi and Yusefzadi, 2015). All morphological evidence in the provided images in the mentioned studies is very similar to the morphological characteristics of the specimens studied in the current investigation. Based on the recent studies *C. sertularioides* f. *farlowii* (Weber Bosse) Børgesen was reported as a synonym with *C. sertularioides* f. *longipes* (J. Agardh) Collins in the algaebase (Guiry and Guiry, 2021).

The morphological characters of the specimens studied in current research are in accordance with the diagnostic characters described for the *Fucus selago* by Turner (1808), which later changed to *Caulerpa* selago by Agardh (1817).

The measured sizes of the whole plant, erect branches, stolon diameter, ramuli size, and forms are very similar to the original description provided for the *Fucus selago* Turner (1808), which later were transferred to the *Caulerpa* genus by Agardh (1817).

Genus *Caulerpa* is a relatively complicated group of the Caulerpaceae family. This complication has led to the nomenclature of too many species, subspecies, varieties, forms, and "ecads" in this genus. Most of these problems are caused by the morphological plasticity made by environmental conditions, which cause many mistakes in nomenclatures that, in turn, misunderstand the field of biodiversity, ecology, biochemical application, etc (Piazzi and Balata, 2009). Phylogenetic analysis as a modern biotechnological device helps overcome complicated issues in the taxonomic studies of different organisms. Many phylogenetic studies on the *Caulerpa* resolved some complex issues in this genus (Fama et al., 2002; Stam et al., 2006; Saunders and Kucera, 2010; Kazi et al., 2013; Sauvage et al., 2013; Draisma et al., 2014; Belton et al., 2014, 2015; Karthick et al., 2020; Zaw et al., 2020).

In the current study, by using a combination of molecular and phylogenetic analyses, we

resolved *C. selago* in the area which, based on morphological characters, has been reported *Caulerpa sertularioides* var. *farlowi* from Abu Dhabi, UAE (Nizamuddin and Gessner, 1970; John, 2005; 2012; John and Al-Thani, 2014); Bahrain (Silva et al., 1996; Rizk et al., 1999), Saudi Arabia (De Clerck and Coppejans, 1994; 1996; Silva et al., 1996), Iran (Sohrabipour and Rabiei, 1999a; 1999b; Sohrabipour et al., 2004; Rabiei et al., 2005; Sohrabipour and Rabiei, 2007, Shams and GhaedAmini, 2017). It should be considered that the record by Nizamuddin and Gessner (1970) was the first mention of the variety from Abu Dhabi at the coastlines of the Persian Gulf. The achieved results showed the presence of *C. selago* species on the southern coasts of Iran. Sequences of Iranian samples well matched with the only one sequence of *C. selago* specimens submitted in genBank (NCBI) from the Egyptian coast of the Red Sea. These results also showed that the tufA gene could help distinguish the members of this genus at the species level and overcome the morphological complication of the genus members.

C. selago is the most common *Caulerpa* species on the southern coastlines of Iran and can grow in extended mats on protected sandy-covered rocks and sandy bottoms in the winter and spring seasons. The results revealed more studies need to do on the taxonomic, ecological, biochemical aspects, and commercial application of the species in this genus.

Acknowledgments

This research was supported by Nogen Company anf Biotechnology and Bioscience Research Center, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

References

Agardh CA. (1817). Synopsis algarum Scandina viae: adjecta disposition universali algarum. pp. [i]-xl, 1-135. Lundae [Lund]: Ex officinal Berlingiana.

Al-Abdessalaam TZ. (2007). Coastal and Marine Habitats. In: Al-Abdessalaam, TZ. (ed.): Marine Environment and Resources of Abu Dhabi. pp. 55-91. Environment Agency, Abu Dhabi and Motivate Publishing.

Basson PW. (1979a). Marine algae of the Arabian Gulf coast of Saudi Arabia (first half). Botanica Marina. 22: 47-64.

Basson PW. (1979b). Marine algae of the Arabian Gulf coast of Saudi Arabia (second half). Botanica Marina. 22: 65-82.

Basson PW. (1992). Checklist of marine algae of the Arabian Gulf. Journal of the University of Kuwait Science. 19: 217-228.

Belton GS, Huisman JM, Gurgel CFD. (2015). Caulerpaceae. In:Huisman, JM. (ed.): Algae of Australia: Marine Benthic Algae of North-Western Australia. 1. Green and Brown Algae, pp. 75-102. Canberra & Melbourne. ABRS & CSIRO Publishing.

Belton GS, Prud'homme van Reine WF, Huisman JM, Draisma SGA, Gurgel CFD. (2014). Resolving phenotypic plasticity and species designation in the morphologically challenging *Caulerpa racemosa–pelta-*

ta complex (Chlorophyta, Caulerpaceae). Journal of Phycology. 50 (1): 32-54.

Chen X, Sun Y, Liu H, Liu S, Qin, Li P. (2019). Advances in cultivation, wastewater treatment application, bioactive components of *Caulerpa lentillifera* and their biotechnological applications. Aquatic Biology. 7: 1-15.

Coppejans E, Prud'homme van Reine WF. (1992). Seaweeds of the Snellius-II expedition (E. Indonesia): the genus *Caulerpa* (Chlorophyta-Caulerpales). Bulletin des séances Académie Royale des Sciences d'Outre-Mer. 37: 667-712.

De Clerck O and Coppejans E. (1994). Status of the macroalgae and seagrass vegetation after 1991 Gulf War oil spill. Courier Forsch-Inst. Senckenberg. 166: 18-21.

De Clerck O and Coppejans, E. (1996). Marine Algae of the Jubail Marine Wildlife Sanctuary, Saudi Arabia. In: Krupp F, Abuzinada AH, Nadar IA. (eds.): A Marine Wildlife Sanctuary for the Arabian Gulf: Environmental Research and Conservation following the 1991 Gulf War Oil Spill. pp. 199-289. NCWCD, Riyadh and Senckenberg Research Institute, Frankfurt.

Diini F. (2013). Opportunities and challenges for developing *Caulerpa racemosa* as functional foods, pp. 85-96. The 1st International Symposium On Aquatic Product Processing.

Dorgham MM. (1990). Distribution of benthic algae in the Qatari Arabian Gulf. Bulletin of the Institute of Oceanography and Fisheries. 16: 49-63.

Draisma SGA, Prud'homme van Reine WF, Sauvage T, Belton GS, Gurgel CFD, Lim PE, Phang SM. (2014). A re-assessment of the infra-generic classification of the genus *Caulerpa* (Caulerpaceae, Chlorophyta) inferred from a time-calibrated molecular phylogeny. Journal of Phycology. 50 (6): 1020-1034.

Fritsch FE. (1965). The Structure and Reproduction of the Algae. Vol. I. Cambridge University Press, London. 791 pp.

Fama P, Wysor B, Kooistra WHCF, Zuccarello GC. (2002). Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tuf*A Gene. Journal of Phycology. 38: 1040-1050.

Gharanjik BM. (2000). The marine algae of the Sistan and Baluchestan Province, Iran. Iranian Journal of Fisheries Science. 2 (2): 57-70.

Guiry MD and Guiry GM. (2021). AlgaeBase. World-Wide Electronic Publication, National University of Ireland, Galway.

Hall TA. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series. 41: 95-98.

John DM. (2005). Marine Plants. In: Hellyer, P. Aspinall, S. (eds.):The Emirates a Natural History. pp. 161-167, 351-352. Trident Press, London, UK.

John DM. (2012). Marine Algae (Seaweeds) Associated with Coral Reefs in the Gulf. In: Riegl BM and Purkis SJ. (eds.): Coral Reefs of the Gulf: Adaptation to Climatic Extremes. pp. 308-335. Springer, Dordrecht.

John DM and Al-Thani RF. (2014). Benthic marine algae of the Arabian Gulf: a critical

review and analysis of distribution and diversity patterns. Nova Hedwigia. 98 (3-4): 341-392.

Karthick P, Murthy KN, Ramesh C, Narayana S, Mohanraju R. (2020). Molecular authentication of green algae *Caulerpa* (Caulerpales, Chlorophyta) based on ITS and *tuf*A genes from Andaman Islands, India. Indian Journal of Experimental Biology. 58 (2): 109-114.

Kazi MA, Reddy, CRK, Jha B. (2013). Molecular phylogeny and barcoding of *Caulerpa* (Bryopsidales) based on the *tuf*A, rbcL, 18S rDNA and ITS rDNA genes. Plos One. 8 (12): 1-13.

Khairuddin K, Sudirman S, Huang L, Kong Z.L. (2020). *Caulerpa lentillifera* polysaccharides-rich extract reduces oxidative stress and pro inflammatory cytokines levels associated with male reproductive functions in diabetic mice. Applied Sciences. 10 (24): 1-14.

Kokabi M and Yousefzadi M. (2015). Checklist of the marine macroalgae of Iran. Botanica Marina. 58 (4): 307-320.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson J.D, Gibson TJ, Higgins DG. (2007). Clustal W and Clustal X version 2.0. Bioinformatics. 23 (21): 2947-2948.

Matanjun P, Mohamed S, Muhammad K, Mustapha NM. (2010). Comparison of cardiovascular protective effects of tropical seaweeds, *Kappaphycus alvarezii*, *Caulerpa* lentillifera, and *Sargassum polycystum*, on high-cholesterol/high-fat diet in rats. Journal of Medicinal Food. 13 (4): 792-800.

Matanjun P, Mohamed S, Mustapham NM, Muhammad K. (2009). Nutrient content of tropical edible seaweeds, *Eucheuma cottonii*, *Caulerpa lentillifera* and *Sargassum polycystum*. Journal of Applied Phycology. 21 (1): 75-80.

Meyer KD and Paul VJ. (1992). Intraplant variation in secondary metabolite concentration in three species of *Caulerpa* (Chlorophyta: Caulerpales) and its effects on herbivorous fishes. Marin Ecology Progress Series. 82: 249-257.

Mshigeni KE and Dorgham M. (1987). Benthic Marine Algae of Qatar. A Preliminary Survey. Unesco regional office, Doha, Qatar, 78 pp.

Najdek M, Korlević, M, Paliaga P, Markovski M, Ivančić I, Iveša L, Felja I, Herndl GJ. (2020a). Dynamics of environmental conditions during a decline of a *Cymodocea nodosa* meadow. Biogeosciences.17: 3299-3315.

Najdek M, Korlević M, Paliaga P, Markovski M, Ivančić I, Iveša L, Felja I, Herndl GJ (2020b). Effects of the invasion of *Caulerpa cylindracea* in a *Cymodocea nodosa* meadow in the northern Adriatic Sea. Frontier in Marine science. 7: 1-16

Niwano Y, Beppu F, Shimada T, Kyan R, Yasura K, Tamaki M, Nishino M, Midorikawa Y. Hamada H. (2009). Extensive screening for plant foodstuffs in Okinawa, Japan with anti-obese activity on adipocytes *in vitro*. Plant Foods for Human Nutrition. 64 (1): 6-10.

Nizamuddin M and Gessner F. (1970). The

Marine Algae of Northern Part of the Arabian Sea and of the Persian Gulf. Gebrueder Borntraeger Publisher. pp. 42.

Piazzi L and Balata D. (2009). Invasion of alien macroalgae in different Mediterranean habitats. Biological Invasions.11: 193-204.

Preez RD, Majzoub ME, Thomas T, Panchal SK, Brown L. (2020). *Caulerpa lentillifera* (sea grapes) improves cardiovascular and metabolic health of rats with diet-induced metabolic syndrome. Metabolites. 10 (12): 1-18

Rabiei R., Assadi M, Nejadsattari T, Majd A, Sohrabipour J. (2005). The study of species diversity in association of *Gracilariasalicorniain* northeast of Qeshm Island. Pajouhesh and Sazandegi. 17 (1): 85-92.

Rizk AM, AL-Easa HS, Korporbst JM. (1999). The Phytochemistry of the Macro and Blue-Green Algae in the Arabian Gulf. The Doha Modern Press Ltd., Qatar. 746 pp.

RohaniGhadekolaei K, Rajabi I, Rameshi H, Dehghani R, Behzadi S., Hossaini MR, Saito H, Xue C, Yamashiro R, Moromizato S, Itabashi Y. (2010). High polyunsaturated fatty acid levels in two subtropical macroalgae, *Cladosiphono kamuranus* and *Caulerpa* lentillifera. Journal of Phycology. 46 (4): 665-673.

Saunders GW and Kucera H. (2010) An evaluation of *rbcL*, *tuf*A, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. Cryptogamie Algologie. 31 (4): 487-528.

Sauvage T, Payri C, Draisma SGA, Prud'homme Van Reine WF, Verbruggen H, Belton GS, Gurgel CFD, Gabriel D, Sherwood AR, Fredericq S. (2013). Molecular diversity of the *Caulerpa racemosa-Caulerpa peltata* complex (Caulerpaceae, Bryopsidales) in New Caledonia, with new Australasian records for *C. racemosa* var. *Cylindracea*. Phycologia. 52 (1): 6-13

Shams M and GhaedAmini N. (2017). A new taxonomic survey of *Caulerpa Lamouroux* species (Chlorophyta, Caulerpales) in the southern coasts of Iran. American Scientific Research Journal for Engineering, Technology, and Sciences (ASRJETS). 36 (1): 45-57.

Silva PC, Basson PW, Moe RL. (1996). Catalogue of the Benthic Marine Algae of the Indian Ocean. Vol. 79. University of California Press. 1259 pp.

Sohrabipour J, Nejadsattari T, Assadi M, Rabiei R. (2004). The marine algae of the southern coast of Iran, Persian Gulf, Lengeh area. Iranian Journal of Botany. 10 (2): 83-93.

Sohrabipour J and Rabiei R. (1996). New records of algae for Persian Gulf and flora of Iran. The Iranian Journal of Botany. 7 (1): 95-115.

Sohrabipour J and Rabiei R. (1999a). A list of marine algae from seashores of Iran (Hormozgan Province). Qatar University Science Journal. 19: 312-337.

Sohrabipour J and Rabiei R. (1999b). A list of marine algae of seashores of the Persian Gulf and Oman Sea in the Hormozgan Province. The Iranian Journal of Botany. 8 (1): 131-162.

Sohrabipour Jand Rabiei R. (2007). The checklist of green algae of the Iranian coast lines of the Persian Gulf and Gulf of Oman. The

Iranian Journal of Botany. 13 (2): 146-149.

Stam WT, Olsen JL, Zaleski SF, Murray SN, Brown KR, Walters LJ. (2006). A forensic and phylogenetic survey of *Caulerpa* species (Caulerpales, Chlorophyta) from the Florida coast, local aquarium shops, and e-commerce: establishing a proactive baseline for early detection. Journal of Phycology. 42: 1113-1124.

Susilowati A, Mulyawan AE, Putri TW. (2019). Antioxidant activity of the sea grape (*Caulerpa racemasa*) used as an antioxidant lotion. Oriental Journal of Chemistry. 35 (4): 1443 -1447.

Swofford DL. (2002). PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0 Beta. Sinauer Associates, Sunderland, Massachusetts.

Tanabe AS. (2007). Kakusan: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. Molecular Ecology Notes. 7: 962-964

Turner D. (1808). *Fuci* sive plantarum fucorum generi a botanicis ascriptarum icons descriptiones et historia. *Fuci*, or coloured figures and descriptions of the plants referred by botanists to the genus *Fucus*. Vol. I. pp. 1-164, pls 1-71.

Van den Hoek C, Mann DG, Jahns HM. (1995). Algae. An Introduction to Phycology. Cambridge University Press, Cambridge. 623 pp.

Weber-van Bosse, A. (1898). Monographie des Caulerpes. Annales du JardinBotanique de Buitenzorg. 15: 243-401.

Zaw NDK, Wiradana PA, Naw SW, Nege AS, Alamsjah MA, Akbar REK, Rosi F. (2020). First report on molecular identification of *Caulerpa* green algae from Mandangin Island Indonesia using Partial 18SrRNA Genes. Journal of Aquaculture and Fish Health. 9 (3): 252-265.

Zheng J, Li Z, Manabe Y, Kim M, Goto T, Kawada T, Sugawara T. (2018). Siphonaxanthin, a carotenoid from green algae, inhibits lipogenesis in hepatocytes via the suppression of liver X receptor α activity. Lipids. 53: 41-52.

Study of Wastewater Treatment by Microalga From Caspian Sea

Ladan Baftehchi[1], Hamid Sobhanian[1*], Neda Soltani[2], Gholam Reza Bakhshikhaniki[1], Mehrouz Dezfolian[3]

Received: 2021-09-11 Revised and accepted: 2021-10-28

Abstract

In today's modern world, using water by man and different industries produces a large volume of effluents and wastewaters that are a serious threat to humans, environments, and ecosystems. Refining and treatment of the wastewaters are essential. This research investigated the potential of marine microalgae *Fischerella* sp. in refining nutrients from wastewater. *Fischerella* sp. was collected from the Caspian Sea. Artificial wastewater was prepared by adding different amounts of NaCl (1, 5%), $CaCl_2$ (35, 100 mg/L), $MgSO_4$ (75, 150 mg/L), $NaNO_3$ (50, 2000 mg/L) and K_2HPO_4 (6, 500 mg/L) to BG110 medium in 12 runs according to Design expert. The growth and chlorophyll contents in various treatments were measured, and nutrient analysis of the medium was performed on the 10th and 20th days after algal culture. Results showed that maximum growth, chlorophyll and decreasing of Ca^{2+}, Mg^{2+}, NO_3^- and PO_4^{3-} content were observed in 1% NaCl, 35 and 100 mg/L $CaCl_2$, 150 mg/L $MgSO_4$, 2000 mg/L $NaNO_3$ and 6, 500 mg/L K_2HPO_4. The most removing activity was shown in the stationary phase of algal growth. Also, in these conditions a decrease in TDS, TOC, and COD was observed. It can be concluded that *Fischerella* sp. is a suitable microalga decreasing nutrients in1% NaCl and the highest amount of N and P.

Keywords: Caspian Sea, Chlorophyll, *Fischerella* sp., Growth, Nutrients, Wastewater treatment

Introduction

Water is one of the most critical needs of humans worldwide. By increasing the population and man's activities like agriculture or industrialization, wastewater and effluents have proliferated, including saline wastewaters. However, water bodies and reservoirs are limited, and climatic changes have affected them directly.

Saline wastewater contains various types and inorganic salts such as NaCl, Na_2SO_4, $MgSO_4$, KNO_3, K_2HPO_4, and $NaHCO_3$ (Kester et al., 1967). There is no evidence or report about the exact amount or kinds of

1-Department of Biology, Payame Noor University (PNU), P.O.Box: 19395-4697, Tehran, Iran
2-Department of petroleum microbiology, Research Institute of Applied Science of ACECR, ShahidBeheshti University. Tehran, Iran.
3- Biotechnology Research Center, Karaj branch, Islamic Azad University, Karaj, Iran.
*Email address: motif3000@yahoo.com

these elements because they depend on the sources that produce them. The significant sources of saline wastewaters are agriculture, industries, and secondary activities. (Hoang Nhat et al., 2018).

Excessive irrigation of saline lands and climatic changes such as lack or untimely rainfall, which are common in recent years, generate saline wastewater. In this case, changes in weather conditions (e.g., excessive heat) that cause more water exploitation should be considered. Mariculture is another activity that is responsible for producing saline wastewaters. Industrial activities such as tanneries, pharmaceutical, textile, petroleum, paper and pulp, and mining produce saline wastewaters with various organic, inorganic, heavy metals, and other contaminants. Another source of saline wastewater is related to the membrane and ion exchange technologies such as Reverse Osmosis (R.O.) in water desalination systems (Hoang Nhat et al., 2019).

Saline wastewater affects the environment seriously. It damages crop production by changing the osmotic balance, so the assimilation of nutrients is disturbed, and therefore growth rate and crop yields are affected. This result was later contradicted by Al-Jaloudet al. (1993). As this wastewater has various elements, it can pollute the drinking water, threatening the health of humans and other users. This wastewater affected the ecology directly and indirectly. It can instantly change the biological characters of flora and fauna of the environment (Alves et al., 2018), but in indirect form, as saline wastewater contains inorganic nutrients, primarily N and P; it is the main factor for eutrophication and is toxic to ecosystems and human beings (Anjuli et al., 2015).

By considering all above mentioned, the treatment of wastewater is essential. There are different methods for treating saline wastewater; biological (Ahmadi et al., 2017; Xiao & Robert, 2010), physicochemical (Aller, 2016; Wen et al., 2018), and hybrid technologies (Nguyen et al., 2018; Tomei et al., 2017). Among these, applying zeolite, using an anaerobic process, membrane process, and halophilic microorganisms (microalgae) process are most used. Most of these methods are costly because saline wastewaters contain a high degree of inorganic salts, but among them, as microalgal processes have no secondary pollutant and besides it can create profit; eco-friendly; they are considered as a cost-effective and green process (Hoang Nhat et al., 2019) (Anjuli et al., 2015).

Microalgae are microscopic photoautotroph organisms. They can produce oxygen using atmospheric CO_2 and solar energy. Cyanobacteria (blue-green algae) are more suitable for wastewater treatment. Cyanobacteria are ubiquitous and, depending on their species, can tolerate various situations with different ranges like saline wastewaters (Anjuli et al., 2015).

These microorganisms have unique characteristics that make them suitable for application in biotechnology, agriculture, industry, and wastewater treatment. They can

grow in a variety type of mediums, even wastewaters. They are photoautotrophs and play an essential role in reducing the greenhouse effect on the environment. Besides oxygen production, some blue-green algae species have nitrogen fixation abilities that help them grow even in low N contents and make them a good candidate as biofertilizers.

Cyanobacterial biomass has many applications in, e.g., feed, pharmaceutical, industries, and biofuel. So cultivation of blue-green algae in wastewater is a safe method for treatment and producing biomass (Subramaniyan 2012; Anjuli 2015).

So in this research, the growth and chlorophyll contents of *Fischerella* sp. that isolated from the Caspian Sea of Iran in artificial wastewater (AWW) were investigated and its potential to remove nutrients in two-phase of growth (logarithmic and stationary phase) was studied.

Material and methods

Sampling and culture

Sampling was performed from different parts of the Caspian Sea; Salmanshahr, Mahmoodabad, Khzarabad (Mazandaran province), and Geisomcoastline (Gillan province) in the north of Iran from August-September 2019. Samples were transferred to the petroleum microbiology lab of ACECR of Shahid Beheshti University and cultured in F/2 medium (Guillard and Ryther,1962) by solidified agar plate (Belcher et al. 1982) and soil culture (Sardeshpande and Goyal, 1981) methods. Cultures were kept in the culture room of ACECR of Shahid Beheshti at 25± 2 ˚C with a Florence lamp and a duration of 8 L/16D.

After a month, the dominant specimens in both cultures were purified. The *Fischerella* sp. was selected as one of the most common specimens. Then mass cultivation is done in liquid cultures with BG110 medium (Kaushik, 1987). Liquid cultures also were kept in the culture room with the above condition. Aeration of samples was done by an aquarium air pump, Artman HP-4000.

Molecular identification

The DNA extraction was done by the Fermentas DNA extraction kit (K0512). Moreover, the molecular identification of specimens was performed by the PCR of the 16s ribosomal region and sequencing of the PCR product by Nubel et al. (2000).

Analysis of growth and chlorophyll contents

Analysis of growth was performed according to the biomass changes by optical density (OD) method in λ 750 nm (spectrophotometer, Light wave WPA) that was done every 2 days for 25 days (Soltani, 2006).

Chlorophyll contents were obtained by preparing methanol extracts of specimens and measuring their density in λ 665 nm according to (1) by Marker's (1972) method.

$$\text{Chl. } (\mu g/ml) = 13.14 \times \text{OD } 665 \text{ nm} \quad (1)$$

Experimental design

The artificial wastewater (AWW) used in this experiment was prepared by dissolving the common nutrients of wastewaters in BG110 medium with minimum and maxi-

Table 1. Designed runs as artificial wastewater in our experiments

Run	NaCl (%)	$CaCl_2$ (mg/l)	$MgSO_4$ (mg/l)	$NaNO_3$ (mg/l)	K_2HPO_4 (mg/l)
1	1.00	35	150	50	6
2	1.00	100	150	2000	500
3	1.00	100	75	2000	6
4	5.00	100	75	50	500
5	5.00	35	75	50	6
6	1.00	35	75	2000	500
7	5.00	100	150	50	6
8	5.00	35	150	2000	6
9	5.00	100	75	2000	500
10	1.00	35	150	2000	6
11	1.00	100	75	50	500
12	5.00	35	150	50	500

mum levels as follows: NaCl (1, 5%), $CaCl_2$ (35, 100 mg/L), $MgSO_4$ (75, 150 mg/L), $NaNO_3$ (50, 2000 mg/L) and K_2HPO_4 (6, 500 mg/L) in 12 runs that were designed by Design expert (Table 1). Our blank was BG110 medium.

The *Fischerella* sp. is cultured under each run's specification and kept in the above culture room. The OD and chlorophyll contents of these 12 runs were also measured every 2 days for 25 days, as in section 2.3.

Analysis of growth medium

The growth medium was filtered with filter paper to study the changes in nutrients. It was analyzed in 3 phases: before adding algal specimens (preliminary study), at the logarithmic phase of algal growth (10^{th} day of cultivation), and during the stationary phase of algal development (20^{th} day of cultivation).

Analysis and removal percentage of cation and anions

In this research, cations: Na^+ (ppm), Ca^{2+} (ppm), and Mg^{2+} (ppm) were analyzed by ICP- OES method.

Cl^- (%) was measured by titration with $AgNO_3$; meanwhile, NO_3^-(ppm) was measured by spectrophotometry UV-visible (Shimadzu) in λ 220 and 270nm. Indeed, PO_4^{3-} (ppm) measured by Standard Method 4500-P-C.

The nutrient removal percentage was calculated by Do et al. (2019) according to equation (2):

$$\% \text{ nutrient removal} = C_1(\text{initial concentration}) - C_2(\text{final concentration})/C_1 \times 100 \quad (2)$$

Other analysis

Other analyses, including Total Dissolved Solid (TDS) (mg/l), was performed by electroconductivitimeter, Total Organic Carbon (TOC) (mg/l as C) by Standard method 5310 B; Chemical Oxygen Demand (COD)

(mg/l as O_2) by Standard method 5220B.

Statistical analysis

Statistical analysis of growth and chlorophyll contents were done for three replication of each samples by ANOVA (SPSS V.24) and also removing nutrients were done by and ANOVA (Design-Expert V.7.0 Softwares).

Results

Growth and chlorophyll contents

Growth curves of *Fischerella* sp. according to the biomass change in 12 run and blank are shown in Fig.1. According to these results, runs 2, 11, 10, and 6 with 1% NaCl had more growth and also had significant difference with the blank sample($p \leq 0.05$). Among them run 2; NaCl 1%, $CaCl_2$ 100, $MgSO_4$ 150, $NaNO_3$ 2000, K_2HPO_4 500 mg/L; had the most growth and its difference with runs 10, 11, 6 was significant($p \leq 0.05$).

Runs with 5% NaCl kept their viability in stationary form, but their growth was noticeably less than the others.

Measuring of chlorophyll contents in 12 runs and blank also showed that the blank (BG110 with no additive nutrients) had the most chlorophyll with significant difference ($p \leq 0.05$) with other samples. Runs with 1% NaCl (10, 1, 11, 6, 2, 3) had the same content of chlorophyll, but among them, the most chlorophyll contents were observed in run 10; NaCl 1%, $CaCl_2$ 35, $MgSO_4$ 150, $NaNO_3$ 2000, K_2HPO_4 6 mg/L. The chlorophyll contents in 5% NaCl decreased significantly.

Sequence analysis

The sequence of the 16S rRNA gene was determined for *Fischerella* sp. ISC 123. The nucleotide sequences described in this study were submitted to NCBI under NCBI's accession number OK594059.

Analysis of nutrients in growth medium

Analysis of cations and anions

Analysis of cations (Na^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^-, NO_3^- and PO_4^{3-}) and removing percent of them from growth media were shown in Table 2. According to the results, it can be concluded that runs 2,

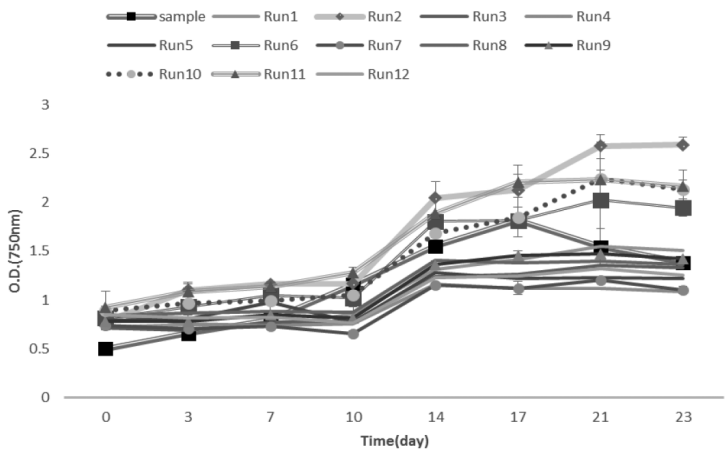

Fig. 1. Growth curve of *Fischerella* sp. in 12 runs according to OD

6, 10, and 11 had the most effective in decreasing cations, especially Ca^{2+} and Mg^{2+}. Results also showed that the most percent of removing anions were observed in run 2. This run removed 98% nitrate from growth media on the 10th day of culture. Runs 1 and 11 could remove significant amounts of nitrate(95,94%) on the 20th day of culture. Run 4 could remove 73.3% Cl^- on the 10th day.

Analysis of TDS, TOC and COD

Analysis of TDS, TOC and COD and their removing percentage from growth media were shown in Table 3. According to the results it can concluded that runs run 7 had the most removal of TDS (69%) and runs 10 and 11 had the most potential in removing TOC and COD.

Discussion

According to the growth results, it can be concluded that salinity is a critical factor in controlling the growth. In this case, *Fischerella* sp. had a noticeable growth in 1% NaCl, and even in some cases, its development was more than the blank (with no added NaCl). Although NaCl 5% was unsuitable for growth, this microalga could tolerate it for two weeks and had a steady growth phase. Our specimen was a marine sample, so it adapted to this condition better.

These results were similar to the research on *Chlorella vulgaris* (freshwater microalgae), *Chlorella* sp., and *Stichococcus* sp. (marine microalgae) with 0.1, 1, 3. 5, and 5% NaCl. In this case, *C. vulgaris*,

Table 2. The removing percentage of cations (Na^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, NO_3^-, PO_4^{3-}) from growth media in 12 runs during the10th and the 20th day of microalga culture

	Na^+ %		Ca^{2+} %		Mg^2+ %		Cl^- %		NO_3^- %		PO_4^{3-} %	
Runs	10th	20th	10th	20th	10th	20th	10th	20th	10th	20th	10th	20th
1	-	-	1.7	-	-	-	27	5.8	-	95	32	3.57
2	-	-	49.7	73	4.6	4	30	4.48	98	-	-	-
3	-	-	5	7.6	-	-	30.5	18.6	-	-	65	68
4	-	-	-	-	-	-	73.3	14.5	-	37	-	-
5	-	-	-	-	-	-	-	12.8	-	-	-	-
6	-	-	47.4	62	8.2	7.3	-	1.56	-	25	-	-
7	-	-	-	-	2.7	-	-	-	-	-	6	-
8	-	-	1.9	-	-	-	-	-	-	20	-	-
9	-	-	-	-	-	-	-	-	3	7	0.43	-
10	-	24.3	-	-	-	-	-	-	-	15	42	-
11	-	-	63.8	85	10.6	7	-	-	21	94	10	-
12	-	-	-	-	-	-	-	13.35	-	28	5.8	-

Table 3. The removing percentage of TDS, TOC and COD from growth media in 12 runs during 10th and 20th day of microalga culture

	TDS %		TOC %		COD %	
Runs	10th	20th	10th	20th	10th	20th
1	-	-	-	-	-	-
2	52	-	-	-	-	-
3	55	45	80	62	65.7	71.4
4	-	-	50.2	35.3	66.1	70.7
5	35.4	29	79	74.5	57.7	61.5
6	48.4	47.5	73.3	71.2	75	65
7	64.3	69.2	-	-	12.5	-
8	-	34	41.5	36.1	75	37.5
9	57	66.5	81.2	74	81.5	75
10	-	-	94.6	82.6	92.1	89.5
11	47.5	52	89.6	56	85	60
12	7	34	50.2	20.2	75	56.2

the freshwater microalgae, has maximum growth and biomass yield in 0.1 salinity. Still, the other marine ones had the most growth and biomass yields at 1%, and by increasing the salinity(3.5 and 5%), their biomass reduced significantly (Hoang Nhat et al., 2018).

Although there are some exceptions depending on the species and the medium conditions, for example, Zhou et al. (2017) explored that *Spirulina platensis* could produce noticeable biomass in 2.24% salinity. It should be considered that other culture conditions such as nutrient concentration, pH, and different light: dark cycles are also responsible in this case. In another study, Kim et al.(2016) also showed that *Acutodesmus obliquus* have suitable biomass in 5.2% salinity. Church et al.(2017), which researched the characteristics of *C. vulgaris* in synthetic saline wastewater, also remarked that higher salinities decrease the biomass content, similar to our findings. Another study on *C. vulgaris* and the effect of NaCl and KCl on its growth by Church et al.(2017) also showed that algal growth would be decreased even lower biomass by increasing salt concentration for both salts production was observed with KCl than NaCl.

Photosynthesis activities include photosystem I (PSI) and PSII in microalgae (Kebede, 1997). PSI relates directly to the chlorophyll contents and their changes. So

studying the chlorophyll contents, especially in stress conditions like salinity and wastewater, is essential. The chlorophyll contents showed that this pigment is too sensitive to increasing salinity because all the runs in 5% NaCl had a significant decrease in chlorophyll. It means that *Fischerella* sp. had low nutrient consumption in this salinity. In 1% NaCl, after a week, chlorophyll production increased, and the difference among the runs related to the other nutrients in the culture medium. These results were similar to *Stichococcus* sp., which produces a significant amount of chlorophyll in 0.1 and 1% salinity, but its contents at 3.5 and 5% were very low (Hoang Nhat et al., 2018). It should be noticed that marine algae usually adapt more to saline stress because their physiological systems are compatible with these conditions. Guetet al.(2012) reported that the increased salinity in *Nanochloropsis oculata* causes culture to decrease the growth rate and pigment contents (e.g., chlorophyll and carotenoids).

Eliminating cations and anions in different runs showed that the highest potential of removing nutrients was observed in runs with high growth and chlorophyll contents (2, 6, 10, 11) in 1% NaCl. So it can be concluded that the amount of elimination relates to the growth of samples.

Studies on *C. vulgaris* in various amounts of NaCl and KCl (0- 1.5- 3 and 4.5%) shows that lower salt concentration causes faster nutrient to remove (ammonia) for both salt type (Church et al., 2017).

Considering the removing percentage in 2 phases (the10^{th} and 20^{th} day) revealed that although both steps were active in removing, the most removing activities were performed at the end of the growth phase (20^{th} day) in most runs.

In this case, research on *Phormidium* sp. showed that this microalga could remove 48 and 30% of PO_4-P and NO_3-N from anaerobically treated swine wastewater (Su et al., 2012). Kamilya et al. (2006) revealed that *Nostoc muscorum* and *Spirulina platensis* have nutrient removal potential from fish culture effluent. In this case, *N. muscorum* can remove 14.17 and 41.8 % of NO_3-N and PO_4-P while *S. platensis* remove 50.39 and 47.76% of NO_3-N and PO_4-P, respectively, in 7 days. Besides microalgal monocultures, Silva-Benavides and Torzillo (2011) use the co-culture of *Planktothrix* sp. and *Chlorella* sp. to treat municipal wastewater. This co-culture can remove 100 and 88% of PO_4-P and NO_3-N. Also, a consortium of filamentous strains (*Phormidium*, *Limnothrix*, *Anabaena*, *Westiellopsis*, *Fischerella*, and *Spirogyra*) was studied and reported to remove 100 and 97 % of nitrate and phosphate (Anjuliet al., 2015). These results indicate that cyanobacterial species can significantly remove nutrients (mainly N and P) from various types of wastewater.

Research on *C. vulgaris*, *Scenedesmus obliquus*, and *Oocystis minuta* by Ajala and Alexander (2019) removing nutrients in wastewater revealed that *C. vulgaris* could reduce 93% nitrate after 14 days of culti-

vation; meanwhile, *O. minute* can consume phosphate up to 95%.

The removal of TOC and COD was also related to growth. Total Organic Carbon (TOC) is a crucial nutrient for microalgae. Hoang Nhat et al. (2018) showed that the highest TOC removal is at 0.1 and 1% salinities. In this case, *C. vulgaris* can remove 92% TOC on the 10th day, and *Stichococcus* sp. can remove more than it at 1% NaCl. El-Bestawy (2008) researched *Anabaena variabilis*, *A. oryzae* and *Tolypothrix ceylonica* about their role in improving water quality in domestic industrial wastewater. They showed that *A. variabilis* had the highest BOD and TDS reduction (89.3 and 39%) while can reduce COD up to 73.6%. Another research by Nagasathya and Thajuddin (2008) on wastewater treatment potential and water quality improvement of paper mill effluent by *Phormidium tenue* was performed, removing 14.5, 17.6, and 45.26% of salinity, BOD, and COD in 20 days.

By considering the results, it can be concluded that marine species have more adaptability to saline conditions. In this case, *Fischerella* sp. is a suitable microalga with good growth and chlorophyll in wastewaters with 1% NaCl and high amounts of nitrate and phosphate. Besides this, its ability to the treatment of wastewater elements is considerable.

Acknowledgment

The authors acknowledge the support from the Research Center of Applied Science of ACECR for lab experiments.

References

Ahmadi M, Jorfi S, Kujlu R, Ghafari S, DarvishiCheshmeh Soltani R, Jaafarzadeh Haghighifard N. (2017). A novel salt-tolerate bacterial consortium for biodegradation of saline and recalcitrant petrochemical wastewater. Journal of Environmental management. 191, 198-208. Doi: 10.1016/j.jenvman.2017.01.010.

AjalaSh, Alexander O. (2020). Assessment of *Chlorella vulgaris*, *Scenedes musobliquus*, and *Oocystis minuta* for removal of sulfate, nitrate, and phosphate in wastewater. International journalof energy and environmental engineering. 11: 311-326. https:// doi.org/10.1007/s40095-019-00333-0.

Al-Jaloud AA, Hussain G, Al-Saati AJ, Karimullah S. (1993). Effect of wastewaters on plant growth and soil properties. Arid land Research and Management. 7 (2): 173-179. Doi: 10.1080/15324989309381346.

Aller MF. (2016). Biochar properties: Transport, fate, and impact. Critical Reviews in Environmental Science and Technology. 46 (14-15): 1183-1296. Doi: 10.1080/10643389.

AlvesRDC, de Medeiros AS, Nicolaus MCM, Neto AP, de Assis Oliveira F, Lima LW, Gratao PL. (2018). The partial root –zone saline irrigation system and antioxidant respomnces in tomato plants. Plant Physiology and Biochemistry. 127: 366-379. Doi: 10.1016/j.plaphy.2018.04.006.

Anjuli S, Nirmal R, Radha P, Amrik Singh Ah. (2015). Cyanobacteria as potential options for wastewater treatment. Phytoremedia-

tion: Management of Environmental Contaminents. 2 . Doi: 10.1007/978-3-319-10969-5-8.

Belcher H and Swale E. (1982). Culturing algae, a guide for school and colleges. Tilus Wilson & son LTD.

Church J, Hwang JH, Kim K T, McLean R, Oh Y K, Nam B, Joo JC, Lee WH. (2017). Effect of salt type and concentration on the growth and lipid content of *Chlorella vulgaris* in synthetic saline wastewater for biofuel production. Bioresource Technology. 243 (Supplement C): 147-153.

Do J, Jo S, Kim I, Na H, Lee JH, Kim HS, Yoon H. (2019). A feasibility study of wastewater treatment using domestic microalgae and analysis of biomass for potential application. Water. 11: 2294.

El-Bestawy E. (2008). Treatment of mixed domestic-industrial wastewater using cyanobacteria. Journal of Industrial Microbiology and Biotechnology. 35: 1503-1516.

Gu N, Lin G, Qin G, Lin J, Huang L. (2012). Effect of salinity change on biomass and biochemical composition of *Nannochloropsis oculata*. Journal of World Aquaculture Society. 43 (1): 97-106.

Guillard RRL and Ryther JH. (1962). Studies on marine planktonic diatoms, *Cyclotella nana* Hustedt and *Detonula confervacae* (Cleve) Gran. Canadian Journal of Microbiology. 8: 229-239.

Hoang NhatPhong V, HuuHao N, Wenshan G, Soon WoongCh, DinhDuc N, ZhuoCh, Xiaochang CW, Rong Ch, XinboZh. (2019). Microalgae for saline wastewater treatment: a critical review. Critical Review in Environmental Science and Technology. Doi: https://doi.org/10.1080/10643389.2019.1656510.

Hoang NhatPhong V, HuuHao N, Wenshan G, Yiwen L, Soon Woong Ch, DinhDuc N, Phuoc Dan N, XuanThanh B, Jiawei R. (2018). Identification of the pollutants' removal and mechanism by microalgae in saline wastewater. Bioresource Technology. Doi: https://doi.org/10.1016/j.biortech.2018.12.026.

Kamilya D, Sarkar S, Maitli TK. (2006). Growth and nutrient removal rates of *Spirulina platensis* and *Nostoc muscurum* in fish culture effluent: a laboratory –scale study. Aquaculture Research. 37: 1594-1597.

Kaushik BD. (1987). Laboratory methods for blue-green algae. Associated publishing company.

Kebede E. (1997). Response of *Spirulina platensis* (*Arthrospira fusiformis*) from lake Chitu, Ethiopia to salinity stress from sodium salts. Journal of Applied Phycology. 9 (6): 551-558.

Kester DR, Duedall IW, Connors DN, Pytkowicz RM. (1967). Preparation of artificial seawater. Limnology and Oceanography. 12 (1): 176-179. Doi: 10.4319/lo.1967.12.1.0176.

Kim HC, Choi WJ, Chae AN, Park J, Kim HJ, Song KG. (2016). Treating high-strength saline piggery wastewater using the heterotrophic cultivation of *Acutodesmus obliquus*. Biochemistry Engineering Journal. 110 (supplement C): 51-58.

Marker AFH. (1972). The use of acetone and methanol in the estimation of chlorophyll

in the presence of phaeophytin. Freshwater Biology. 2 (4): 361-385.

Nagasathya and Thajuddin N. (2008). Decolourization of paper mill effluent using hypersaline cyanobacterium. Research Journal of Environmental Sciences. 2: 408-414.

Nguyen NC, Chen SS, Nguyen HT, Chen YH, Ngo HH, Guo W, Le QH. (2018). Applicability of an integrated moving sponge biocarrier-osmotic membrane bioreactor M.D. system for saline wastewater treatment using highly salt-tolerant microorganisms. Seperation and Purification Technology. 198: 93-99. Doi: 10.1016/j.seppur.2017.01.011.

Nubel U, Garcia-Pichel F, Clavero E, Muyzer G. (2000). Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. Environmental Microbiology. 2: 217-226.

Sardeshpande JS and Goyal SL. (1981). Distribution pattern of blue-green algae in rice field soils of Konkan region of Maharashtra state. Phykos. 20 (1-2): 102-106.

Silva-Benavides AM, Torzillo G. (2011). Nitrogen and phosphorous removal through laboratory batch cultures of microalgae *Chlorella vulgaris* and cyanobacterium *Planktothrix isothrix* grown as monoalgal and co-cultures. Journal of Applied Phycology. 24: 267-276

Soltani N, Khavari-Nejad RA, Tabatabaie M, Shokravi Sh,Valiente EF. (2006). Variation of nitrogenase activity, photosynthesis and pigmentation of cyanobacterium *Fischerella ambigua* strain FS18 under different irradiance and pH. World Journal of Microbiology and Biotechnology. 22: 571-576.

Su Y, Mennerich A, Urban B. (2012). Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. Bioresource Technology. 124: 157-162.

Subramaniyan V. (2012). Potential application of cyanobacteria in industrial effluents- a review. Bioremediation and Biodegradation. Doi: 10.4172/2155-6199.1000154.

Tomei MC, MoscaAngelucci D, Stazi V, Daugulis AJ. (2017). On the applicability of a hybrid bioreactor operated with polymeric tubing for biological treatment of saline wastewater. Science of The Total Environment. 599: 1056-1063. Doi: 10.1016/j.scito-tenv.2017.05.042.

Wen J, Dong H, Zeng G. (2018). application of zeolite in removing salinity/sodicity from wastewater: A review of mechanisms, challenges and opportunities. Journal of Cleaner Production. 197: 1435-1446. Doi: 10.1016/j.clepro.2018.06.270.

Xiao Y and Roberts DJ. (2010). A review of anaerobic treatment of saline wastewater. Environmental Technology. 31 (8-9): 1025-1043. Doi: 10.1080/09593331003734202.

Zhou W, Li Y, Gao Y, Zhao H. (2017). Nutrients removal and recovery from saline wastewater by *Spirulina platensis*. Bioresource Technology. 245 (part A): 10-17.

Study of Stress-Responsive Genes Effective on Lipid Profiling in Some Newly Isolated Cyanobacteria

Sara Kabirnataj[1], Ahmad Farhad Talebi[2*], GhorbanAli Nematzadeh[3], Prashant Singh[4]

Received: 2021-09-23 Revised and accepted: 2021-11-17

Abstract

In this study, 17 heterocystous cyanobacterial strains were investigated for biomass density, lipid content, lipid productivity, and fatty acid composition. A superior strain for biofuel production was selected for a further study evaluating the lipid enhancement under some environmental stress including different concentrations of NaCl, H_2O_2, and $CuSO_4$. Moreover, Real-time PCR analysis determined the dependency of cyanobacterial cell age and also stressed conditions on the expression variations of some essential genes in lipid biosynthesis pathways, and photosynthesis. Among the studies strains, *Aliinostoc* sp. produced the highest chlorophyll (19.79 µg/mg DW) and lipid (12.64% DW) content, therefore it was selected to optimize experimental conditions for lipid biosynthesis; The optimal conditions for lipid production ($CuSO_4$:3 µm, NaCl:10 mM, H_2O_2:0) resulted in an increase in lipid (12.82%) and a decrease in chlorophyll (10.32%) content, compared to the control condition. These results were confirmed by up-regulation of the *acc*D gene (73%) as the first gene involved in the lipid production pathway, and down-regulation of the *rbc*L gene (54%), which is an indicator of photosynthetic rate. Since the ability of growth and lipid production of *Aliinostoc* sp. has been optimized under salinity and heavy metal stress conditions, lipid production could simultaneously perform by biorefining of contaminated water resources.

Keywords: Pollution; Wastewater Treatment; Lipid; Cyanobacteria; Stress

Introduction

Cyanobacteria, one of the first oxygenic photosynthetic prokaryotes, play a critical role in the evolution of life through the build-up of oxygen in the Earth's atmosphere (Komárek 2013). Their high capacity in fixing atmospheric carbon and nitrogen makes cyanobacteria highly resistant under extremely undesirable conditions. Moreover, unique morphological and physiological characteristics empower cyanobacteria to survive and make populations in

1-Oilseed Research and Development Company, Sari, Iran
2-Department of Biotechnology, Faculty of New Sciences and Technologies, Semnan University, Semnan, Iran
3-Genetic and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University, Sari, Iran
4-Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, India
*E-mail address: aftalebi@semnan.ac.ir

almost all habitats; it is a significant advantage over photosynthetic organisms such as plants (Asadian et al., 2018). There have been numerous reports on the isolation of cyanobacteria from undesirable growth conditions such as stringent saline, alkaline or acidic medium, and heavy metal-contaminated soils and waters (Cellamare et al., 2018; Heidari et al., 2018). Unfortunately, anthropogenic activities' nonstop exploitation of natural resources has led to an uncontrolled increase in environmental pollution such as heavy metals. This phenomenon is more dominant in developing countries since more than 80% of sewage is discharged untreated into polluted rivers, lakes, and coastal areas (UNESCO, 2019). Developing innovative techniques to treat sewage effectively can further protect water resources and guarantee the quality of foods produced by aquatic ecosystems. Several physical and chemical methods have been used for heavy metal remediation; however, the non-biological approaches are costly due to the issues of high costs and stringent rules for input chemicals, incomplete removal of the ions, and prerequisites for particular instruments. It is well documented that many cyanobacteria can efficiently detoxify heavy metal-contaminated waters (Singh et al., 2019). They have large cell surface area, unique cell wall compositions such as different multifunctional groups, and superior mucilage volume with high binding affinity simple nutrient requirements (Metallothionine, Phytochellatine and, polyphosphate) (Ghorbani et al., 2022). Cyanobacteria uptake metal ions in two different modes; a) passive uptake, heavy metal ions are entrapped onto the binding sites present in the cellular structure, b) active uptake, the living cells transit heavy metals into the cell through the cell membrane, expending energy to keep moving against a gradient (Kumar et al., 2015). It should note that some heavy metals are vital in small amounts for various biochemical and physiological processes of the cells. Trace elements such as iron (Fe), molybdenum (Mo), magnesium (Mg), and copper (Cu) act as micronutrients in the cells whilst they will suppress biological activities in higher concentrations. Copper, for example, is essential for several metalloenzymes and electron transport activities, whereas at high concentration, it leads to photosynthesis and respiration repression, cell division inhibition, and cell death initiation in photosynthetic organisms (Goswami et al., 2015; Ahad and Syiem, 2018). Trace elements are applied in biochemical engineering techniques for lipid over-production by regulating involved metabolic pathways. For example, Battah et al. (2015) reported that supplementation of 2.5 µM of cobalt nitrate resulted in a 22% increase in lipid productivity compared to the controls in *Chlorella vulgaris*. Disruption of algal photosynthetic machinery due to the interruption of plasma membrane function could eventually lead to the accumulation of lipids as secondary storage products in favor of carbohydrates. Magnesium was also attributed to the reprogramming of green

algae metabolism by Huang et al. (2014). They reported that lipid productivity of the *Monoraphidium* sp. was increased by 18% over the controls using 100 μM Mg^{2+} supplementation. These results were also confirmed by Dong et al. (2020), who nearly duplicated the biomass and lipid productivity by 800 μM Mg^{2+} supplementation in the medium of green microalgae.

Moreover, lipid accumulation of *Scenedesmus* sp. was significantly increased up to 31% with the addition of Pb^{2+} up to 1 mg/L (Pham et al., 2020). As an essential constituent for several enzymes related to the lipid synthesis pathway, iron has also been reported numerously as a crucial element for algal cell growth and lipid enhancement (Kong et al., 2020). Environmental stresses such as heavy metal induction force algal cells to shift their lipid synthesis pathway to neutral lipids production, most of which is triacylglycerol (TAG). The cell can withstand adverse environmental conditions (Sharma et al., 2012; Antoni et al., 2021). The first step in the fatty acid (FA) synthesis pathway is the carboxylation of acetyl-CoA, which is accomplished by the acetyl-CoA carboxylase (ACC) enzyme (Post-Beittenmiller et al., 1992). The beta carboxyl transferase subunit of ACC is codded by the acetyl-CoA carboxylase beta subunit (*acc*D) gene. So, *acc*D is the first gene involved in FA synthesis, and its correlation with lipid biosynthesis has been reported numerously. Kumar et al. (2017) investigated the regulation of the *acc*D gene in some cyanobacterial strains. They noted that the changes in the lipid content and the *acc*D gene expression were observed under different nitrogen to phosphate ratios. *Acc*D expression was significantly correlated with lipid accumulation; overexpression of the *acc*D gene under stress conditions could lead to higher lipid accumulation in the cells (Talebi et al., 2014; Che et al., 2017). This study aimed to evaluate optimized cyanobacterial growth conditions for enhanced lipid biosynthesis under saline and heavy metal stress conditions. Incubation in such conditions stimulates the cells to simultaneously produce value-added byproducts (e.g., lipid) during the depletion of contaminations (e.g., CO_2 and contaminated wastewater).

Moreover, the characterization of the physiological and molecular responses is also considered. The *acc*D gene expression was investigated as the first gene involved in the FA synthesis pathway to achieve the purpose. The expression of the desaturase B (*des*B) gene was studied to evaluate the desaturation rate of FAs. Finally, to survey the possible responses of photosynthesis-related cell activities, the expression of the *rbc*L gene was also analyzed. These results could throw light on lipid metabolism at the molecular level using transcriptionally profiling the regulated genes.

Material and methods

Cyanobacterial strains and growth condition

Seventeen heterocystous cyanobacterial strains were isolated from different rice

Table 1. List of studied cyanobacterial strains and crossRefs for better identification

Strain code	Genus	16SrRNA accession number (NCBI)	Source of isolation	Reference
SA4	*Calothrix* sp.	MK956202	Rice field, Lamizdeh, Amol, Mazandarn. N36^{O}32.153/E052^{O}19.284	(Kabirnataj et al., 2019)
SA5	*Desmonostoc* sp.	MF770266	Rice field, Dohezar, Tonekabon, Mazandaran. N36^{O}44.521/E050^{O}50.426	(Kabirnataj et al., 2019)
SA9	*Aliinostoc* sp.	MK503790	Rice field, Neka, Mazandaran. N36^{O}38.364/E053^{O}18.299	-
SA10	*Desikachary aconstricta*	MK354274	Rice field, Neka, Mazandaran. N36^{O}38.364/E053^{O}18.299	(Kabirnataj et al., 2020)
SA16	*Neowestiellopsis bilateralis*	MF066911	Rice field, Dohezar, Tonekabon, Mazandaran. N36^{O}44.521/E050^{O}50.426	(Kabirnataj et al., 2018)
SA18	*Aliinostoc magnakinetifex*	MK503791	Rice fiels, Semeskandeh, Sari, Mazandaran N36^{O}33.970/E053^{O}08.924	(Kabirnataj et al., 2020)
SA20	*Aliinostoc* sp.	MK956203	Rice field, Siahkola, Noor, Mazandaran N36^{O}32.695/E052^{O}06.234	-
SA22	*Aliinostoc* sp.	MK956204	Rice field, Semeskandeh, Sari, Mazandaran N36^{O}35.548/E053^{O}13.855	-
SA24	*Aliinostoc catenatum*	MK503792	Rice field, Semeskandeh, Sari, Mazandaran N36^{O}33.970/E053^{O}08.924	(Kabirnataj et al., 2020)

SA28	*Aliinostoc* sp.	MK956205	Rice field, Amirabad, Behshahr, Mazandaran. N36°47.308/E053°20.994	-
SA30	*Aliinostoc constrictum*	MK503793	Rice field, Ojaghsar, Babolsar, Mazandaran. N36°40.813/E052°36.364	(Kabirnataj et al., 2020)
SA33	*Neowestiellopsis persica*	MF066912	Rice field, Kordekheyl, Chalous, Mazandaran. N36°39.494/E051°24.113	(Kabirnataj et al., 2018)
SA35	*Aliinostoc* sp.	MK956206	Rice field, Sefidtameshk, Ramsar, Mazandaran. N36°57.495/E050°35.781	-
SA43	*Aliinostoc* sp.	MK503794	Rice field, Goharbaran, sari, Mazandaran. N36°48.905/E053°11.042	-
SA45	*Aliinostoc* sp.	MK956207	Rice field, Darayakouchak, Miankaleh, Mazandaran. N36°47.414/E053°32.793	-
SA46	*Aliinostoc* sp.	MK503795	Rice field, Miandoroud, Sorak, Mazandaran. N36°35.518/E053°13.866	-
SA47	*Calothrix* sp.	MK956208	Rice field, Neka, Mazandaran. N36°38.364/E053°18.299	(Kabirnataj et al., 2019)

fields located in the northern part of Iran, Caspian Sea borders, in January 2013 (Table 1). The samples were grown in the sterile liquid BG110 medium (Stanier et al., 1979), the pH of the medium adjusted to 7.2, and cultures were maintained in a culture room under the illumination of approximately 50 $\mu molm^{-2}s^{-1}$ with a photoperiod of 14/10 h light/dark cycle at 23 C. Details of morphological and molecular characterization of the strains were earlier reported (Kabirnataj et al., 2018; 2019; 2020). Measurement of pigments, lipid, and other physiological parameters was performed when the culture reached maximal density after about 21 days.

Growth kinetic parameters

Chl a quantification

Chlorophyll extraction was carried out according to Sinetova et al. (2012) with minor modifications as follows: 500 µl of deionized water was added to 20 mg of freeze-dried biomass and set aside for 15 minutes to absorb moisture. 9.5 ml methanol was added to the mixture and vortexed for 30 seconds. After 24 hours keeping at 4° C in the dark condition, the samples were vortexed again, centrifuged at 4 °C for 20 min at 10,000 rpm. The optical density of the supernatant read at 665 nm and 720 nm. Chlorophyll a (Chl a) concentration (µM) finally was measured by the following equation (Eq. 1):

$$\text{Chl a } [\mu g/ml] = 14.4 (A_{665} - A_{720}) \quad \text{(Eq. 1)}$$

Total lipid content

Total lipid extraction was performed on 21-day-old culture using the adopted protocol from Bligh and Dyer (1959) in 3 replicates. 100 mg (W) dried biomass (freeze-dried) were added to the glass tube and 4 ml of distilled water. Then 10 ml of methanol and 5 ml of chloroform were added, vortexed for 30 seconds, and kept overnight in the dark on the shaker. After 12 hours, 5 ml of water and 5ml of chloroform were added, vortexed, and finally centrifuged at 5000 g for 10 minutes. The lower phase was transferred to the pre-weighed glass tube (W_1) and dried at 40 °C. The tubes were weighed (W_2) after drying, and the lipid content was calculated by the weight difference method using equation number 2:

$$\text{Lipid content (\%DW)} = (W_2 - W_1 / W) \times 100 \quad \text{(Eq.2)}$$

Biomass and lipid productivity determination

Table 2. Experimental range and levels of actual and coded factors of Box–Behnken design used in RSM in terms

Independent variables	Design variable	Range and levels		
		Low (-1)	Medium (0)	High (+1)
$CuSO_4$ (µM)	A	0	1.5	3
NaCl (mM)	B	0	5	10
H_2O_2 (µM)	C	0	1.5	3

The biomass productivity was determined by calculating the difference in dry biomass weight (W_1) on the first day (D_1) and after 21 days of cultivation (W_2 and D_2).

Biomass productivity ($g.l^{-1}.day^{-1}$) = ($W_2 - W_1$)/(D_2-D_1) (Eq. 3)

Lipid productivity was calculated according to the following equation (Eq. 4):

Lipid productivity ($mg.l^{-1}.day^{-1}$) = Biomass productivity ($g.l^{-1}.day^{-1}$) × Lipid content (%DW) × 1000 (Eq. 4)

GC analysis

GC analysis of all 17 strains was carried out on the 21st day after cultivation based on the method reported by Talebi et al. (2013). The FAs profile was determined by the direct transesterification method, a single-step extraction, and a derivatization process. The FA designation was done with an RTX-wax column (30 m, 0.25 mm, 0.25 μm). The oven temperature was set at 120 °C for 3 min, increased to 220 °C at a rate of 20 °C/min, and kept for 30 min. The quantitative method was carried out with standard external mixtures of FAs (37-Component FAME Mix, Sigma, USA) and was run earlier under similar conditions.

Experiment design and stress induction

A cyanobacterial strain with the highest chlorophyll, lipid content, and desired FAs composition was selected for further analysis. Three different abiotic stresses were investigated on lipid production by applying different concentrations of $CuSO_4$, NaCl, and H_2O_2 using Design Expert Software Version 10 (Stat-Ease Inc., Minneapolis, USA). The Box-Behnken experimental design (BBD) under the response surface methodology (RSM) was applied to estimate the main interactions and optimal conditions. Chlorophyll and lipid content were studied as dependent output. Experiments were statistically designed at three coded levels, low (−1), medium (0), and high (+1), which corresponds to $CuSO_4$ (0, 1.5, 3 μM), NaCl (0, 5, 10 mM), and H_2O_2

Table 3. Sequences of the primer pairs for real-time PCR. *sec*A is a housekeeping gene, and *acc*D, *rbc*L and *des*B genes represent the biochemical activities of the cells

Primer name	Amplicon length (bp)	Sequence (5-3)	Reference
*sec*A	F — 150 — R	GCCGAAATGAGAACCGGGGAAG / GAAACGGTGTACCTGCCCCATC	(Szekeres et al., 2014)
*acc*D	F — 119 — R	ATGGCAAACAACGAAGAATC / CACAGTCCATCAGCAATTTC	Present study
*rbc*L	F — 161 — R	GGTATCCACGTATGGCATATG / CCTTCGTTACGAGCTTGAA	Present study
*des*B	F — 147 — R	GCAAGGAACGATGTTTTGGG / TGACTAATACGCCAACCGTG	Present study

(0, 1.5, 3 μM) respectively, with 5 replicates at the center point for experimental error estimation (Table 2). Seventeen treatments (Table 6) were applied to one-week-old culture during a 14-day growth. Finally, the biomass was harvested at the end of the 21st day of cultivation, and chlorophyll and lipid content were determined.

The predicted optimum condition (POC) was carried out in three replicates to verify the optimized condition and determine the accuracy and validity of the model. Finally, the actual results were compared to the predicted ones.

Candidate genes and primer design

The expression of three selected genes generally responsive to the studied treatments was evaluated to predict the status of the cells in response to the chemical stresses. *rbc*L, *acc*D, and *des*B were considered the primary regulating genes involved in photosynthesis and lipid synthesis in the cells. Moreover, secA was selected as a housekeeping gene. The sequences from different genera of cyanobacteria were selected from NCBI (https://www.ncbi.nlm.nih.gov) database as well as Cyanobase (http://genome.microbedb.jp/cyanobase), to design primers sequences were aligned with Mega 5 software. Finally, primers were designed from conserved regions using Oligo software (Table 3).

RNA extraction and cDNA synthesis

Gene expression analysis was only carried out on the biomass cultivated on the POC; three biological replicates of BG110 culture as control and the stress-induced samples were applied simultaneously to investigate the expression of the *acc*D, *des*B, and *rbc*L genes. Two ml of cell suspension was harvested at 24 h, 7, and 14 days after stress induction and, due to high RNA degradation, directly used for RNA extraction using Trizol Reagent kit (Cat. No:15596026). To increase the efficiency of RNA extraction, previously crushed and sterilized crushed lamellae glass powders and extraction buffer were added to the samples. All extracted

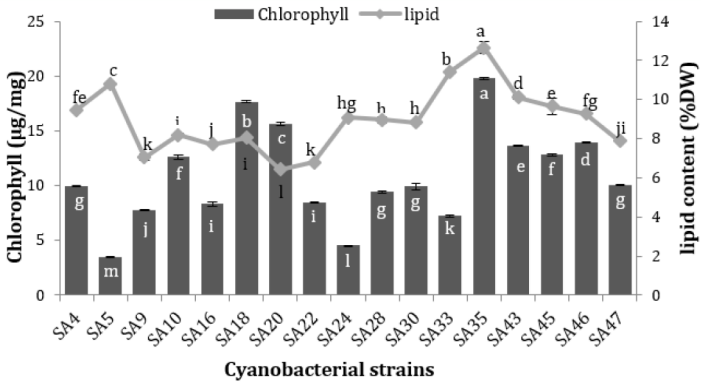

Fig. 1. Chl a and lipid content of cyanobacterial strains measured after 21st day of cultivation.

Values with the letters in the same series showed insignificant differences at P≤0.01

Table 4. Biomass productivity, lipid content, and lipid productivity of 21 days old culture

Cyanobacterial strain	Biomass productivity mg l^{-1} day^{-1}	Lipid content (%DW)	Lipid productivity mg l^{-1} day^{-1}
SA4	5.89	9.47 ± 0.058^{fe}	0.56 ± 0.003^{k}
SA5	8.82	10.81 ± 0.1^{c}	0.95 ± 0.009^{e}
SA9	9.16	7.05 ± 0.18^{k}	0.65 ± 0.017^{j}
SA10	13.53	8.02 ± 0.08^{i}	1.11 ± 0.012^{d}
SA16	10.5	7.7 ± 0.02^{j}	0.81 ± 0.003^{g}
SA18	9.89	8.05 ± 0.05^{ji}	0.8 ± 0.005^{gh}
SA20	10.8	6.42 ± 0.09^{l}	0.69 ± 0.01^{i}
SA22	7.17	6.8 ± 0.1^{k}	0.49 ± 0.007^{l}
SA24	8.48	9.07 ± 0.06^{hg}	0.77 ± 0.005^{h}
SA28	16.12	8.97 ± 0.15^{hg}	1.45 ± 0.025^{b}
SA30	8.8	8.82 ± 0.1^{h}	0.78 ± 0.009^{h}
SA33	10.91	11.43 ± 0.2^{b}	1.25 ± 0.003^{c}
SA35	14.93	12.46 ± 0.31^{a}	1.89 ± 0.046^{a}
SA43	9.16	10.13 ± 0.11^{d}	0.93 ± 0.01^{e}
SA45	9.02	9.67 ± 0.41^{e}	0.87 ± 0.038^{f}
SA46	10.16	9.26 ± 0.03^{fg}	0.94 ± 0.004^{e}
SA47	5.19	7.88 ± 0.06^{ji}	0.41 ± 0.004^{m}

Data expressed the average of three replicates (means± st. dev.). Means of lipid content and lipid productivity are compared using one-way ANOVA and those with different letters are significantly different ($P<0.01$).

RNA was treated with RNase-free DNaseI (Thermo Scientific, Cat. No:EN0521), and finally, first-strand cDNA synthesis was performed by cDNA synthesis kit (Thermo Scientific, Cat. No:K1622), following mentioned instructions.

Gene expression analysis

Expression analysis of selected genes was done with three biological and three technical replicates using ten-fold diluted cDNAs as a template and SYBR Green Real-time PCR Master Mix (ThermoFisher Cat.No:K0222). PCR reactions were performed in a BioRad real-time machine (model: CFX96) using primers shown in Table 2. The amplification was carried out using 35 cycles with a Tm of 52 ˚C for all reactions. One cycle consisted of 30 s at 95 ˚C, 30 s at 52 ˚C, and 45 s at 72 ˚C, and a final extension at 72˚C for 5 min. Delta C_t values (ΔC_t) were calculated using secA as a housekeeping gene, and delta C_t ($\Delta\Delta C_t$) values were used to calculate the relative fold gene expression at specified intervals (Livak and Schmittgen, 2001).

Table 5. Comparison of fatty acids (FAs) profiles produced by seventeen different cyanobacterial strains. Values are given as percent (%) of total detected FAs.

Strains	FAs (%)										SFA	USFA	
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3 (n-3)	C18:3 (n-6)	C22:0	(%)	MUFA (%)	PUFA (%)
SA4	Nd	2.21±0.44	29.67±0.9	2.13±0.17	6.03±1.4	4.76±0.8	7.06±0.11	1.91±0.85	6.88±0.5	-	37.92	6.9	15.85
SA5	0.11±0.5	0.42±0.1	25.2±0.65	8.31±0.47	4.29±0.7	8.46±0.6	15.1±1.03	11.64±0.89	-	-	30.03	16.78	26.75
SA9	Nd	1.7±0.5	nd	16.46±0.69	3.67±0.5	15.82±0.4	19.84±0.9	8.01±0.65	-	-	5.38	32.3	27.86
SA10	0.33±0.4	0.77±0.8	48.28±0.78	8.01±0.7	3.55±0.55	2.75±1.5	2.58±0.4	1.89±0.1	-	-	51.97	10.76	4.47
SA16	0.23±0.86	0.47±0.2	26.93±0.25	27.67±0.2	1.8±0.74	14.93±0.5	6.28±0.4	0.28±0.6	-	-	29.45	42.61	6.57
SA18	0.3±0.15	0.81±0.2	34.20±0.42	11.81±0.75	1.75±0.29	7.11±0.7	5.93±0.67	6.5±0.3	-	-	37.08	18.92	12.44
SA20	0.14±0.18	0.53±0.7	39.06±0.54	11.05±1.2	1.02±0.88	4.11±0.99	6.3±0.81	10.15±0.19	0.01±0.04	-	40.76	15.17	16.48
SA22	0.45±0.92	0.48±0.8	28.21±0.34	15.19±0.4	2.22±0.49	8.28±0.76	22.48±0.81	6.79±0.11	-	-	31.37	23.48	29.28
SA24	0.64±0.2	0.51±0.71	33.88±0.71	12.31±0.22	1.96±0.8	11.15±0.1	7.88±0.23	4.92±0.1	-	-	37.01	23.47	12.81
SA28	0.08±0.61	0.44±0.52	32.32±0.62	13.24±0.73	1.32±0.9	7.5±0.08	15.12±0.4	12.39±1.4	-	-	34.18	20.75	27.52
SA30	0.54±0.54	0.38±0.89	28.57±0.38	15.46±0.82	2.53±0.9	5.37±0.39	3.81±0.5	6.19±0.9	0.04±0.01	0.12±0.01	32.16	20.84	10.05
SA33	0.17±0.43	0.93±0.12	20.22±0.28	32.77±0.98	1.48±0.66	15.74±0.21	6.57±0.3	0.36±0.86	-	-	22.83	48.52	6.93
SA35	0.12±0.76	0.19±0.6	27.11±0.9	8.79±0.31	1.15±0.51	3.1±0.28	8.81±0.78	24.43±0.7	-	2.19±0.05	30.78	11.89	33.25
SA43	0.16±0.7	0.83±0.9	27±0.8	17.05±0.53	0.93±0.1	9.47±0.32	7.95±0.66	5.8±0.89	-	-	28.95	26.53	13.76
SA45	0.15±0.9	0.67±0.8	33.45±0.27	5.54±0.75	3.16±0.15	3.55±0.7	10.38±0.42	7.55±0.7	-	-	37.46	9.09	17.93
SA46	Nd	0.48±0.91	29±0.7	16.31±0.33	1.74±0.7	4.08±0.61	15.46±0.91	10.96±39	-	-	31.23	20.4	26.43
SA47	0.76±0.97	0.82±0.8	27.99±0.7	5.28±0.21	2.31±0.8	5.69±0.91	5.92±0.99	2.79±0.74	6.97±0.08	-	31.89	10.98	15.69
Average	0.3	0.75	30.63	13.38	2.41	7.76	9.85	2.78	7.21	1.15	-	-	-

SFA: saturated FAs; USFA: unsaturated FAs; MUFA: monounsaturated FAs; PUFA: polyunsaturated FA

Results

Selection of putative strain based on chlorophyll assay and lipid analysis

Three growth kinetic parameters i.e., chl a biomass production and lipid content measured in 17 different cyanobacterial strains on the 21st day of cultivation to choose the most promising strain in terms of lipid productivity(Talebi et al., 2013; Li et al., 2017). These data have been presented in Figure 1 and Table 4.

Discussion

Cyanobacteria are a great source of biological products. Among a wide variety of biologically active compounds that produced by cyanobacteria, cyanotoxins are l lines.

The results of the Chlorophyll assay in 17 samples showed that *Aliinostoc* sp. SA35, with Chlorophyll of 19.79 µg/mg, produced the highest chlorophyll content and the second top record in terms of biomass productivity. *Aliinostoc* sp. SA35, with the highest lipid content (12.64 %DW), showed a significant increase in total lipid productivity (1.89 $mg.l^{-1}.day^{-1}$). The results showed that the highest biomass producers did not correspond to the top lipid producers. For instance, strain SA28 with the highest biomass productivity harbors lipid content in the median range. This phenomenon was reported numerously (Nascimento et al., 2013; Ma et al., 2014; Anahas and Muralitharan, 2018). The values obtained for lipid productivity significantly varied from 0.49 to 1.89 $mg.l^{-1}.day^{-1}$ in the studied strains.

In the present study, GC analysis of the cyanobacterial FAs composition indicated that they are highly diverse in FAs values. As seen in Table 5, the highest amount of SFAs (51.97%) was detected in *Desikacharya* sp. SA10 and the lowest total SFAs (5.39%) belonged to *Aliinostoc* sp. SA9. The highest and lowest MUFAs were measured in strains SA33 and SA4, respectively. On average, C16:0 composed about 30.63% of the total FA profile and was seen as the dominant FA in cyanobacterial cells, despite C12:0 composed only about 0.3% of the detected FAs profile. As a polyunsaturated fatty acid (PUFA), alpha-linolenic acid (ALA, C18:3 n-3) belonging to the omega-3 FAs was detected in all the strains, but the highest percentage of 24.43% was seen in *Aliinostoc* sp. SA35. Accordingly, *Aliinostoc* sp. SA35 was selected as a superior strain to investigate FA desaturation rate and related gene expression regulation.

Effect of biochemical engineering on lipid quantity and quality of Aliinostoc sp. SA35

Fitting of response surface models

In this study, RSM based on BBD was used to investigate the effect of independent variables (NaCl, H_2O_2, $CuSO_4$) on response performance (chl a and lipid content) to predict and optimize conditions for lipid and chlorophyll production in *Aliinostoc* sp. SA35 sample. Seventeen experiments, including twelve-star points and five replicates of the center points, were applied to achieve this goal. Based on the results, lipid content varied from 10.4% to 16.2% of dry weight, and chl a recorded from 14.5

Table 6. Design matrix and data for observed and predicted responses from BBD

Run	Level of independent variable			Lipid (%)		Chlorophyll (µg/mg)	
	$CuSO_4$ (µM)	NaCl (mM)	H_2O_2 (µM)	Observed	Predicted	Observed	Predicted
1	1.5	5	1.5	13.5	13.42	23.21	23.59
2	0	10	1.5	15.2	15.27	25.99	26.04
3	1.5	0	3	14.6	14.54	19.7	19.71
4	0	5	3	13.6	13.6	21.02	21.15
5	3	0	1.5	13.2	13.2	21.9	21.85
6	3	10	1.5	11.4	11.36	17.68	17.82
7	1.5	10	3	10.4	10.38	16.5	16.32
8	1.5	5	1.5	13.4	13.42	24.72	23.59
9	0	0	1.5	13.75	13.86	24.4	24.26
10	3	5	0	13.2	13.24	19.37	19.24
11	1.5	5	1.5	13.5	13.42	22.91	23.59
12	0	5	0	15.6	15.53	23.28	23.25
13	3	5	3	11.2	11.31	14.5	14.53
14	1.5	10	0	16.2	16.25	22.01	21.99
15	1.5	5	1.5	13.4	13.42	23.67	23.59
16	1.5	5	1.5	13.5	13.42	23.45	23.59
17	1.5	0	0	12.5	12.51	20.68	20.85

µg/mg to 25.99 µg/mg. Experiment 14th ($CuSO_4$ 1.5 µM and NaCl 10 mM) provided the highest lipid content, while the second experiment (NaCl, 10 mM and H_2O_2, 3 µM) led to maximum chlorophyll production. Moreover, the a and lipid content values were very close to the levels suggested by the software. It indicates the high accuracy of the model in estimating the response variable values (Table 6).

The final responsibility for lipid (Y_1) and chlorophyll (Y_2) content in terms of coded factors are represented in equations:

Y_1=13.42-1.14A-0.11B-0.96C-0.81AB +0AC-1.97BC

Y_2=23.59-2.65A-0.56B-1.7C-1.45AB-0.65AC-1.13BC

ANOVA was performed to estimate the statistical significance of the predicted model considering the calculated p-values. The proposed models for chlorophyll and lipid are significant. It means that the models were appropriate for use in this study. Moreover, the p-value of lack of fit is substantial; it should be non-significant to signify the model; in this study, the p-value of lack of fit of the lipid and chlorophyll content were 0.156 and 0.956 ($p > 0.05$), respectively. All these results indicate that the model is appropriate to predict lipid

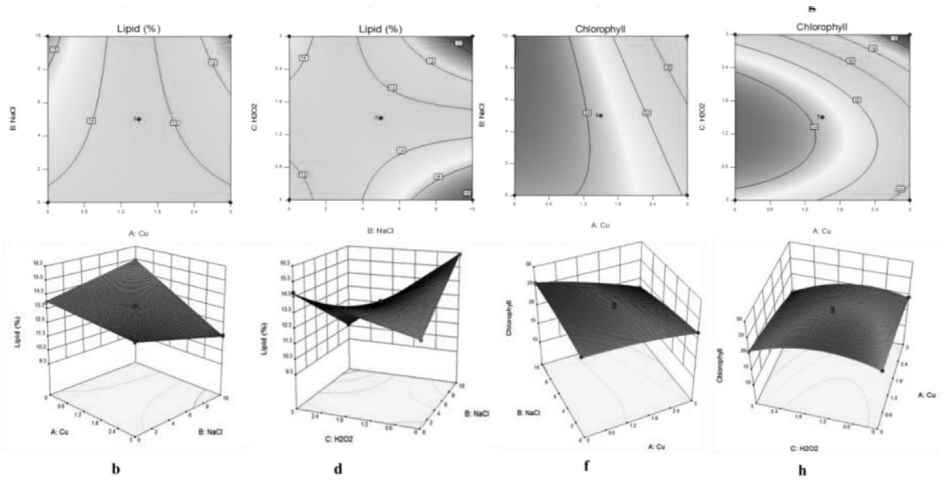

Fig. 2. Three-dimensional response plots and two-dimensional contour plots for lipid (%DW) and chlorophyll content (µg/mg). a,b) interactions of A ($CuSO_4$ µM) and B (NaClmM) and c,d) interaction of C (H_2O_2 µM) and B (NaCl mM) on lipid content enhancement. e,f) interactions of A ($CuSO_4$ µM) and B (NaClmM) and g,h) interaction of A ($CuSO_4$ µM) and C (H_2O_2 µM) on chlorophyll content enhancement. The third factor in all plots is determined in the middle range

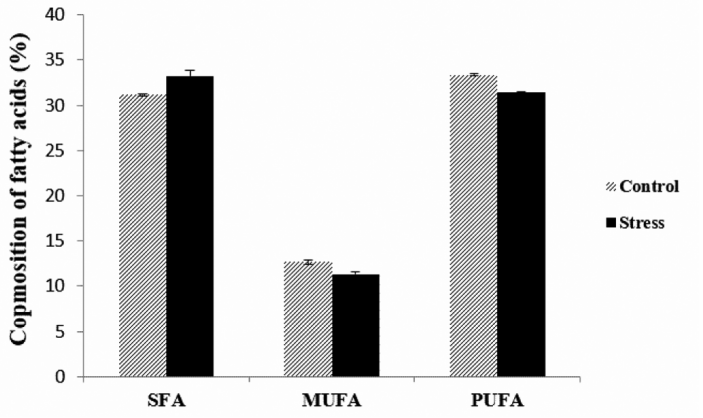

Fig. 3. Percentage contribution comparison of SFA, MUFA, PUFA of *Aliinostoc* sp. SA35 strain in control ($BG\text{-}11_0$) and POC (10 mM NaCl, 3 µM $CuSO_4$ and 0 µM H_2O_2)

production and chlorophyll content within given variables. On the other hand, the value of the correlation coefficient, R^2 (99.82% for lipid and 98.66% for chlorophyll content), shows that the regression models provide an accurate description of the experimental data; these values clarify that the applied model explains 99.82% of the total variation of the lipid response and only 0.18% is explained by the residue. Moreover, low values of the coefficient of variation (CV; 0.6–2.52) provide good evidence for the high precision and reliability of the experiments.

ANOVA results show the significance of all parameters except AC in lipid content and

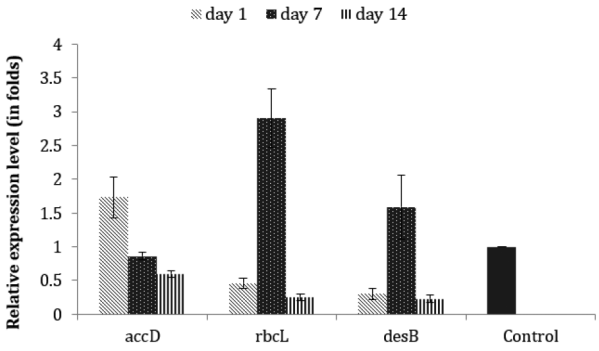

Fig. 4. Expression of *acc*D, *rbc*L and *des*B genes in *Aliinostoc* sp. SA35 strain exposed to POC treatment for 1, 7 and 14 days. Values were normalized to levels of *sec*A, a housekeeping gene

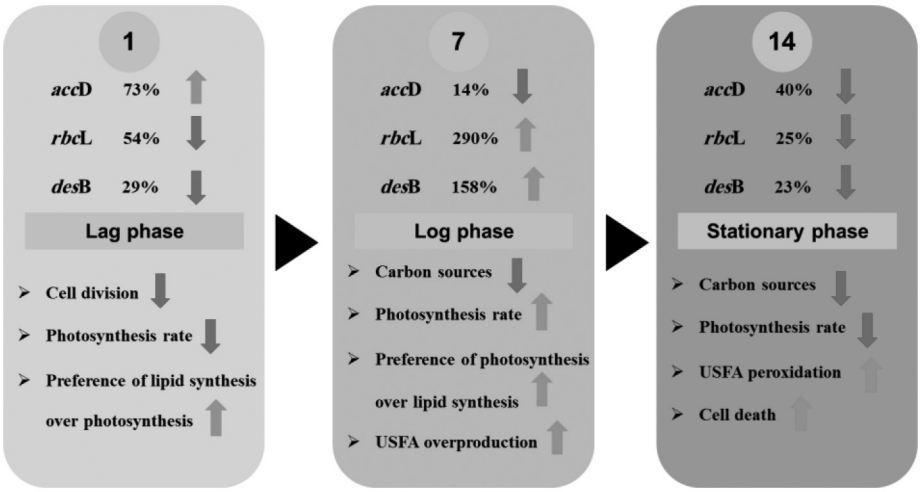

Fig. 5. The *acc*D, *rbc*L and *des*B genes expression variation under POC during two weeks (dayc1, 7 and 14). Blue arrows: up-regulation; Red arrows: down-regulation. (Expression percentage is calculated compared to the control)

B^2 in chlorophyll content. In other words, among all treatments, only the effect of interaction between $CuSO_4$ and H_2O_2 on lipid content enhancement and NaCl (in second-order) on chlorophyll increment was insignificant. Three-dimensional response plots and two-dimensional contour plots for lipid (Fig2 a-d) and chl a (Fig 2e-h) are illustrated in Figure 2 as it is shown, *Aliinostoc* sp. SA35showed an increase of lipid content by NaCl concentration increment; on the other hand, low concentration of $CuSO_4$ and absence of H_2O_2 had a positive effect on lipid content enhancement.

Model optimization

A solution was performed by the Design Expert software (Ver. 10) to determine the optimum condition, in which the concentration of H_2O_2 was adjusted to be varied in the studied range. At the same time, lipid and chlorophyll content was assumed to be at their maximum. Moreover, as the strain

Aliinostoc sp. SA35 could simultaneously refine the wastewater and produce lipid (unpublished data), so the environmental stresses such as NaCl and $CuSO_4$ concentration were adjusted to the maximum value. The RSM model predicts that a medium containing 10 mM NaCl, 3 µM $CuSO_4$ and 0 µM H_2O_2 will provide leading lipid and chlorophyll production of 14.03-14.57 (%DW) and 15.96-19.89 (µg/mg DW), respectively. The cultivation was carried out in triplicates under the optimum conditions to confirm the predicted responses. Based on the results, the mean lipid and chlorophyll content values were 14.5±0.06% (%DW) and 18.01± 0.83 (µg/mg DW), respectively, which means the difference between the predicted and observed values are negligible.

Consequently, the developed model is considered accurate and reliable for optimizing the medium to achieve a high yield of lipid and chlorophyll content. Among the factors investigated to understand the increased lipid production, NaCl caused minor damage to cyanobacterial cells while stimulating cells to synthesize more lipids and resulting in higher viability. Generally, lipid production in the given level was 16.18% more than the control, while chlorophyll production was decreased by 9.36%.

Fatty Acid profile variation

FAs composition, specifically MUFA and PUFA content, define the biodiesel fuel qualities. Therefore after applying POC to the *Aliinostoc* sp. SA35, samples were subjected to GC analysis, and their profiles were analyzed to investigate the changes in the FAs profile and the ratio variation of saturated and unsaturated FAs. As shown in Fig (3), POC induction led to a 6.7% increase in accumulated SFAs and a 7% decrease in UFAs compared to the control.

Effect of biochemical engineering on gene expression

To investigate the dependence of lipid biosynthesis on ACC enzyme expression under stress conditions, the expression of the *acc*D gene was studied. Moreover, the expression of *des*B and *rbc*L genes were also considered under POC conditions to illustrate the effect of abiotic stress on FA profile and photosynthetic machinery (Figure 4, 5). The results showed that expression of the *acc*D gene was increased by 73%, 24h after stress induction. Simultaneously, *rbc*L and *des*B genes were highly downregulated compared to the control in the same condition.

The *des*B gene, responsible for the unsaturation of linoleic acid (C18:2) to alpha-linolenic acid (C18:3), showed an increase in expression on the seventh day by about 158% compared to the control. In comparison, *acc*D decreased about 14% after one week.

Discussion

In this study, strain SA35, a filamentous heterocystous cyanobacterium from the genus *Aliinostoc*, was selected as the most potent strain based on lipid content, lipid productivity, chlorophyll, and ALA content. This strain was further studied for inves-

tigation of the effect of heavy metals and salinity on lipid accumulation. Moreover, optimum conditions for lipid production and cell growth were detected simultaneously. Physiological properties such as chlorophyll content allowed the identification of strains with a higher photosynthetic capability to achieve more biomass productivity. Lipid productivity is an essential factor for biodiesel production influenced by biomass productivity and lipid content. Based on the obtained results, studied strains showed different capabilities in lipid accumulation. It represents the essential importance of cyanobacterial strains screening and evaluation of genetic variation for lipid productivity. Lipid content alone does not allow for proper strain selection since faster-growing species may show lipid productivity greater than those with high lipid content. Moreover, using a native strain with a high growth rate will ultimately reduce production costs as a high growth rate leads to an increase in biomass productivity and finally increases overall yield (Griffiths and Harrison, 2009). On the other hand, biochemical properties should also be considered selecting a potent strain. For example, biodiesel properties such as its lubricity, stability, and cold flow properties are affected by different parameters, mainly FAs composition of the oil (Sorate and Bhale, 2015; Sierra-Cantor and Guerrero-Fajardo, 2017). Length and saturation degree of FAs are two crucial factors in FAs composition detected by GC. On average, palmitic acid (C16:0) was seen as the main FA in the studied strains. C16:0 has also been reported as the main FA in cyanobacterial strains and is considered a dominant FA methyl ester presented in biodiesel (Oliveira et al., 2018). Unsaturated FAs are the main factors determining biodiesel cold flow properties, so a higher content of unsaturated FAs in the oil used in biodiesel production leading superior cold flow properties; therefore, FA methyl esters with longer carbon chain and a higher degree of double bonds show higher lubricity properties (Sorate and Bhale, 2015). ALA, belonging to the group of UFAs, also has nutritional value; it has been confirmed to exert neuroprotective, anti-inflammatory, and antidepressant properties and is required for normal health, especially for the brain development and function (Blondeau et al., 2015) so, it was determined as a critical FA for strain selection. Therefore, *Aliinostoc* sp. SA35 with the highest Chl a content, lipid productivity and ALA FA was selected for further analysis.

Using "salt-out-strategy" microalgae accumulate high salt concentrations by keeping the internal ion concentration low which makes them one of the most promising microorganisms to play in this win-win approach; using saline water resources and producing biomass. The Positive effect of salinity and heavy metal stress on algal and cyanobacterial lipid accumulation has been reported by other studies (Rocha et al., 2019; Chen et al., 2019; Almutairi et al., 2020; Gour et al., 2020; Tiwari et al., 2020). In summary, among different studied treatments to increase lipid production as well

as maintain cell viability and high biomass production, salinity stress using sodium chloride, stimulates cells to synthesize more lipids, causes the most minor damage and keeps higher survival rate in cyanobacterial cells. For instance, around 27% lipid content increment was reported when *Leptolyngbya* sp. cultured at BG11 supplemented with 25 mM sodium chloride (Tiwari et al., 2020). Moreover, an investigation of the effect of salinity on the *Scenedesmus* spp. Showed enhanced lipid production under a 10-15 g/l NaCl (Rocha et al., 2019).

On the other hand, studies show that metal ions in minimal amounts are critical for proper cell function. They act as components of electron-transport proteins in photosynthesis (Cu, Fe), the center of photosynthetic water-oxidation (Mn), or as a significant component of vitamins as well as metalloenzymes (Sunda et al., 2005). However, high amounts of these metals and unnecessary heavy metals (Hg, Sa, Cd, Pb, Cr) cause adverse effects on cells by disrupting photosynthesis, stopping cell division, and enzymatic activities (Monteiro et al., 2012). Studies show that lipid content in microorganisms could increase in small and controlled amounts of these micronutrients. For example, a survey conducted in 2014 on six species of *Chlorella* sp. showed that the highest amount of biomass production is achieved in the presence of 4 mg/L of copper (Sibi et al., 2014).

Moreover, several studies have proven the crucial role of oxidative stress as a mediator in lipid accumulation in photosynthetic microorganisms by applying hydrogen peroxide in appropriate concentrations. Our study showed that the toxic effect of H_2O_2 on elevated lipid accumulation was almost obtained in the lowest concentration in each treatment. In 2014, Yilancioglu et al. (2014) studied the accumulation of lipid in three strains of *Dunaliella salina*, *D. tertiolecta*, and *Chlamydomonas reinhardtii* under hydrogen peroxide treatment. The results showed that the application of oxidative stress by the addition of H_2O_2 increases the production and accumulation of lipids; Depending on the resistance threshold of the studied strains and C/N ratio, lipid production increased up to 44% higher than the corresponding controls. The lethal effect of hydrogen peroxide and its potent oxidizing agent, i.e., extremely reactive hydroxyl radicals (OH-), on the cell membrane, proteins and DNA have been explained by the findings of Battah et al. (2015), and Li et al. (2011). They described that hydrogen peroxide stress might compel microalgae to accumulate considerable amounts of triacylglycerols as a storage lipid under oxidative stress. In other words, the cells decide to reprogram their biosynthetic pathways to further the formation and accumulation of energy storage bio compounds rather than structural ones under stress conditions that then serve as compounds. In summary, stimulation of lipid accumulation under stress conditions in microalgal cells might be attributed to changes in biosynthetic pathways towards the production and accumulation of high-density energy storage compounds

instead of the formation of biomolecules required for cell growth.

Chl a content variation was also an important factor in determining photosynthetic cell viability as stress conditions such as salinity could increase neutral lipid content in microalgae cells and decrease photosynthesis rate to minimize ROS accumulation adverse side effects, simultaneously. Based on the results, chl a was more sensitive to chemical stresses than lipid content. $CuSO_4$ at low concentration and NaCl in high range lead to increased chl a content in *Aliinostoc* sp. SA35. The toxic effect of copper on photosynthetic cells has been reported numerously. Akbarnezhad et al. (2020) demonstrated that phycobiliprotein levels and therefore chl a content decreased in *Spirulina platensis* after copper, zinc, and iron treatment. The inhibitory effect of copper on the growth of *Nostoc muscorum* was also determined by chl a concentration detection. The copper of 10μM lead to a decrease in chlorophyll concentration by about 40% on day seventh (Ahad and Syiem, 2018). Among the factors investigated to understand the increased lipid production, NaCl caused the minor damage to cyanobacterial cells while stimulating cells to synthesize more lipids and resulting in higher viability.

POC induction led to SFAs increase and UFAs decrease in this study. Supporting our evidence, studies show that the induction of heavy metals often leads to a reduction in unsaturated FAs and an increase in the saturated FAs in plants (Upchurch, 2008). Heavy metal stress causes ROS production, including O_2^-, OH, and H_2O_2 which rapidly attacks all biological molecules such as lipids, thus eventually leading to cell dysfunction and death (Chandra et al., 2015). It has been reported that Arsenite (As_2O_3) causes a slight increase in lipid content of *Nannochloropsis* sp. while the amount of USFAs was decreased, SFAs increased, and vice versa (Sun et al., 2015). In the case of copper in our investigation, it should be noted that copper is an essential cofactor, and it is imperative for several metalloenzymes to contain one or more copper atoms, cuproenzymes. Copper, with an ability to alternate between cuprous Cu (I) and cupric Cu (II) oxidation state, acts as an ideal biological cofactor, especially for processes in which the electron transfer is a crucial factor, such as photosynthesis and respiration. However, the two oxidation states of copper allow its participation in essential redox reactions and catalyze the production of ROS, which leads to severe damage to cytoplasmic molecules such as lipids (Huertas et al., 2014). Therefore, this phenomenon would ultimately lead to the peroxidation of UFAs of the plasma membrane, which has been reported numerously. For instance, the SFA and MUFA content of microalga *Nannochloropsis oculata* increased when supplemented with copper at a concentration higher than 0.1 $mmol.L^{-1}$. On the other hand, PUFA content was decreased when the concentration of copper was higher than 0.15 $mmol.L^{-1}$ (Martínez-Macias et al., 2019).

*Acc*D is the first gene in the lipid synthesis pathway, and increased expression of this

gene may indicate an increase in lipid production 24 hours after POC induction in *Aliinostoc* sp. SA35 (Fig. 4). The expression of the *acc*D gene was probably enhanced by the preferential mechanism of lipid accumulation to photosynthesis and cell proliferation. As previously discussed, POC condition could interrupt plasma membrane function leading to disruption of photosynthetic machinery (decreased *rbc*L gene expression). During the favor of carbohydrates production and resultant stopped-cell proliferation, lipid accumulation might be enhanced to increase secondary storage product in the cells due to changes in biosynthetic pathways (increased *acc*D gene expression). It is noteworthy that the *rbc*L and *acc*D genes encode subunits of a multi-domain enzyme and the expression of the enzyme requires the involvement of genes involved in the synthesis of all subunits of the enzyme, so decreasing or increasing the expression of one gene does not necessarily lead to an increase in the desired enzyme product. In the present study, the *des*B gene expression decreased, increased, and again decreased in the first, seventh,, and fourteenth days of stress induction. As noted earlier, increased expression of desaturase genes, which eventually leads to increased UFAs, increases the cell's resistance to stress. However, it should be noted that despite *des*B gene overexpression on day 7, C18:3 FA levels decreased after 14 days compared to the control, which may be due to increased ROS and eventually oxidation of FAs under heavy metal stress conditions during the second week of treatment (Baryla et al., 2000). The *rbc*L gene expression variation indicates photosynthesis and carbon fixation changes in stress conditions. On the other hand reverse behavior of *acc*D gene expression confirmed the preferential mechanisms of cyanobacterial cells for synthesis and storage of lipid in the face of stress. Expression pattern of *des*B gene and variation in FAs profiles pointed out the effect of stress and *des*B gene on FA unsaturation and cell viability power.

Studies show that increasing UFAs in the face of stress leads to increased membrane fluidity and enhanced protection of photosystems I and II and ultimately increased photosynthesis (Sui et al., 2010). This phenomenon has been confirmed by up-regulation of *des*B and *rbc*L genes after 7 days in POC condition. Therefore, increasing UFAs leads to cyanobacterial resistance to non-biological stresses such as salinity. However, this study concludes with two factors, including copper and salt, and it is not possible to study their direct effects on gene expression patterns separately. Previous reports indicate that heavy metals may lead to the oxidation of lipids, and the object of the lipid peroxidation might be UFAs (Baryla et al., 2000; Morsy et al., 2012; Sytar et al., 2013). Therefore, it is likely that the expression of *des*B decreased by day 14 due to the lipid oxidation increment and cell death. Numerous studies have shown that membrane lipids are the frontline to contact stress and are oxidized faster and more quickly as this process modifies membrane properties such as membrane fluidity, a critical physical fea-

ture known to modulate membrane protein localization and function (De La Haba et al., 2013; Sytar et al., 2013). Therefore as cyanobacterial lipids are often membrane type (cytoplasmic and thylakoid membranes lipids), this makes the lipids of these microorganisms more vulnerable to stress.

Based on the results, *Aliinostoc* sp. SA35 could overproduce lipids and grow under 10 mM NaCl and 3 µM $CuSO_4$. POC induction has led to increased lipids to 16.18% and a decrease in the chlorophyll by 9.36%, which was consistent with the findings of the molecular studies, in which the optimal treatment caused a 73% increase in the expression of the *acc*D gene as the first gene involved in the lipid production pathway and 54% decrease in *rbc*L gene expression, which is an indicator of photosynthesis rate. Since the growth and lipid synthesis of the studied strain has been optimized and confirmed under salinity and copper stress, the lipid can be produced simultaneously with the use and bioremediation of contaminated water sources.

Bio-refinery of contaminated water resources is among the unique abilities of algal strains, which could further preserve the freshwater resources during lipid production for industrial applications. Although the presence of heavy metal ions in the growth media limits the use of biomass for biological applications, biofuel production from wastewater-grown cyanobacteria through hydrothermal treatment could overcome this problem. Hydrothermal treatment is a promising route for the valorization of biomass. It efficiently separates more than 90% of heavy metal ions such as copper from the resultant liquid oil (Li et al., 2020).

Acknowledgments

The authors thank the Sari Agricultural Sciences and Natural Resources University, Genetic and Agricultural Biotechnology Institute of Tabarestan, for financial support for accomplishing the present research.

References

Ahad RIA, Syiem MB. (2018). Copper and cadmium-induced toxicity on the cyanobacterium *Nostoc muscorum* Meg 1: a comparative study. EurAsian Journal of BioSciences. 12: 333-45.

Akbarnezhad M, Shamsaie Mehrgan M, Kamali A, Javaheri baboli M. (2020). Effects of microelements (Fe, Cu, Zn) on growth and pigment contents of *Arthrospira* (*Spirulina*) *platensis*. Iranian Journal of Fisheries Sciences. 19: 2020.

Almutairi AW, El-Sayed AEKB, Reda MM. (2020). Combined effect of salinity and pH on lipid content and fatty acid composition of *Tisochrysis lutea*. Saudi Journal of Biological Sciences. 27: 3553-8.

Anahas AMP and Muralitharan G. (2018). Characterization of heterocystous cyanobacterial strains for biodiesel production based on fatty acid content analysis and hydrocarbon production. Energy Conversion and Management. 157: 423-37.

Antoni JS, Daglio Y, Areco MM, Rodríguez MC. (2021). Zinc-induced stress on cells of *Halamphora luciae* (Bacillariophyceae). Eu-

ropean Journal of Phycology. 56:37-50.

Asadian M, Fakheri BA, Mahdinezhad N, Gharanjik S, Beardal J, Talebi AF. (2018). Algal Communities: An Answer to Global Climate Change. CLEAN–Soil, Air, Water.

Baryla A, Laborde C, Montillet JL, Triantaphylidès C, Chagvardieff P. (2000). Evaluation of lipid peroxidation as a toxicity bioassay for plants exposed to copper. Environmental Pollution. 109:131-135. doi: 10.1016/S0269-7491(99)00232-8.

Battah M, El-Ayoty Y, Abomohra AEF, El-Ghany SA, Esmael A. (2015). Effect of Mn^{2+}, Co^{2+} and H_2O_2 on biomass and lipids of the green microalga *Chlorella vulgaris* as a potential candidate for biodiesel production. Annals of Microbiology. 65:155-162. doi: 10.1007/s13213-014-0846-7.

Bligh EG and Dyer WJ. (1959). A rapid method of total lipid extraction and purification1. Canadian Journal of Biochemistry and Physiology. 37. doi: https://doi.org/10.1139/o59-099.

Blondeau N, Lipsky RH, Bourourou M, Duncan MW, Gorelick PB, Marini AM. (2015). Alpha-linolenic acid: An omega-3 fatty acid with neuroprotective properties - Ready for use in the stroke clinic? BioMed Research International. 519830:1-8. doi: 10.1155/2015/519830

Cellamare M, Duval C, Drelin Y, Djediat C, Touibi N, Agogué H, Leboulanger C, Ader , Bernard C. (2018). Characterization of phototrophic microorganisms and description of new cyanobacteria isolated from the saline-alkaline crater-lake Dziani Dzaha (Mayotte, Indian Ocean). FEMS Microbiology Ecology.94:1–25. doi: 10.1093/femsec/fiy108.

Chandra K, Salman A, Mohd A, Sweety R, Ali KN .(2015). Protection Against FCA Induced Oxidative Stress Induced DNA Damage as a Model of Arthritis and In vitro Anti-arthritic Potential of Costus speciosus Rhizome Extract. International Journal of Pharmaceutical and Phytopharmacological Research. 7 (2):383-389.

Che R, Huang L, Xu JW, Zhao P, Li T, Ma H, Yu X. (2017). Effect of fulvic acid induction on the physiology, metabolism, and lipid biosynthesis-related gene transcription of *Monoraphidium* sp. FXY-10. Bioresource Technology. 227:324-334. doi: 10.1016/j.biortech.2016.12.017.

Chen HH, Xue LL, Liang MH, Jiang JG. (2019). Sodium azide intervention, salinity stress and two-step cultivation of *Dunaliella tertiolecta* for lipid accumulation. Enzyme and Microbial Technology. 127:1–5. doi: 10.1016/j.enzmictec.2019.04.008.

De La Haba C, Palacio JR, Martínez P, Morros A. (2013). Effect of oxidative stress on plasma membrane fluidity of THP-1 induced macrophages. Biochimica et Biophysica Acta-Biomembranes. 1828:357–364. doi: 10.1016/j.bbamem.2012.08.013.

Dong X, Huang L, Li T, Xu JW, Zhao P, Yu X. (2020). The enhanced biomass and lipid accumulation in algae with an integrated treatment strategy by waste molasses and Mg2+ addition. Energy Sources, Part A Recovery, Utilization, and Environmental Effects. 42:1183–1192. doi: 10.1080/15567036.2019.1602227.

Ghorbani E, Nowruzi B, Nezhadali M, Hekmat A. (2022). Metal removal capability of two cyanobacterial species in autotrophic and mixotrophic mode of nutrition. BMC Microbiology. 22(58):1-15. https://doi.org/10.1186/s12866-022-02471-8.

Goswami S, Diengdoh OL, Syiem MB, Pakshirajan K, Kiran MG. (2015). Zn(II) and cu(II) removal by *Nostoc muscorum*: A cyanobacterium isolated from a coal mining pit in Chiehruphi, Meghalaya, India. Canadian Journal of Microbiology. 61:209–215. doi: 10.1139/cjm-2014-0599.

Gour RS, Garlapati VK, Kant A. (2020). Effect of Salinity Stress on Lipid Accumulation in *Scenedesmus* sp. and *Chlorella* sp.: Feasibility of Stepwise Culturing. Current Microbiology. 77:779-785. doi: 10.1007/s00284-019-01860-z.

Griffiths MJ and Harrison StL. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology. 21:493–507. doi: 10.1007/s10811-008-9392-7.

Heidari F, Riahi H, Aghamiri MR, Zakeri F, Shariatmadari Z, Hauer T. (2018). 226Ra, 238U and Cd adsorption kinetics and binding capacity of two cyanobacterial strains isolated from highly radioactive springs and optimal conditions for maximal removal effects in contaminated water. International Journal of Phytoremediation. 20: 369-377. doi: 10.1080/15226514.2017.1393392.

Huang L, Xu J, Li T, Wang L, Deng T, Yu X. (2014). Effects of additional Mg^{2+} on the growth, lipid production, and fatty acid composition of *Monoraphidium* sp. FXY-10 under different culture conditions. Annals of Microbiology. 64:1247-1256. doi: 10.1007/s13213-013-0768-9.

Huertas MJ, López-Maury L, Giner-Lamia J, Sánchez-Riego AM, Florencio FJ. (2014). Metals in cyanobacteria: Analysis of the copper, nickel, cobalt and arsenic homeostasis mechanisms. Life 4:865–886.

Kabirnataj S, Nematzadeh GA, Talebi AF, Saraf A, Suradkar A, Tabatabaei M, Singh P. (2020). Description of novel species of *Aliinostoc*, *Desikacharya* and *Desmonostoc* using a polyphasic approach. International Journal of Systematic and Evolutionary Microbiology. 70:3413-3426. doi: 10.1099/ijsem.0.004188.

Kabirnataj S, Nematzadeh GA, Talebi AF, Tabatabaei M, Singh P. (2018). *Neowestiellopsis* gen. nov, a new genus of true branched cyanobacteria with the description of *Neowestiellopsis persica* sp. nov. and *Neowestiellopsis bilateralis* sp. nov., isolated from Iran. Plant Systematics and Evolution. 304:501-510. doi: 10.1007/s00606-017-1488-6.

Kabirnataj S, Nematzadeh GA, Talebi AF, Tabatabaei M, Singh P. (2019). Identification of some heterocystous cyanobacteria isolated from rice fields of Mazandaran province with emphasis on multi-genic approach. Journal of Microbial World. 12: 213-228.

Komárek J. (2013). Modern classification of cyanobacteria. In: Sharma NK, Rai AK, Stal LJ (ed) Cyanobacteria. John Wiley and Sons, Ltd, Chichester, UK. pp: 21-39.

Kong F, Ren HY, Zhao L, Nan J, Ren NQ, Liu BF, Ma J. (2020). Semi-continuous lipid production and sedimentation of *Scened-*

esmus sp. by metal ions addition in the anaerobic fermentation effluent. Energy Conversion and Management. 203:112216. doi: 10.1016/j.enconman.2019.112216.

Kumar R, Biswas K, Kumar Singh P, Kumar Singh P, Elumalai S, Shukla P, Pabbi S. (2017). Lipid production and molecular dynamics simulation for regulation of *acc*D gene in cyanobacteria under different N and P regimes. Biotechnol. Biofuels. 10: 94. doi: https://doi.org/10.1186/s13068-017-0776-2.

Kumar SK, Dahms HU, Won EJ, Lee JS, Shin KH. (2015). Microalgae - A promising tool for heavy metal remediation. Ecotoxicology and Environmental Safety. 113:329-52. doi: 10.1016/j.ecoenv.2014.12.019. Epub 2014 Dec 19. PMID: 25528489.

Li H, Watson J, ZhangY, Lu H, Liu Z. (2020). Environment-enhancing process for algal wastewater treatment, heavy metal control and hydrothermal biofuel production: A critical review. Bioresource Technology. 298:122421. https://doi.org/10.1016/j.biortech.2019.122421.

Li J, Parkefelt L, Persson KM, Pekar H. (2017). Improving Cyanobacteria and Cyanotoxin Monitoring in Surface Waters for Drinking Water Supply. Journal of Water Security. 3:1-8. doi: 10.15544/jws.2017.005.

Li Y, Chen YF, Chen P, Min M, Zhou W, Martinez B, Zhu J, Ruan R. (2011). Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresource Technology. 102:5138–5144. doi: 10.1016/j.biortech.2011.01.091

Livak KJ and Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25:402–408. doi: 10.1006/meth.2001.1262.

Ma Y, Wang Z, Yu C, Yin Y, Zhou G. (2014). Evaluation of the potential of 9 *Nannochloropsis* strains for biodiesel production. Bioresource Technology. 167:503–509. doi: 10.1016/j.biortech.2014.06.047

Martínez-Macias M del R, Correa-Murrieta MA, Villegas-Peralta Y, Dévora-Isiordia GE, Álvarez-Sánchez J, Saldivar-Cabrales J, Sánchez-Duarte RG. (2019). Uptake of copper from acid mine drainage by the microalgae *Nannochloropsis oculata*. Environmental Science and Pollution Research. 26:6311-6318. doi: 10.1007/s11356-018-3963-1

Monteiro CM, Castro PML, Malcata FX. (2012). Metal uptake by microalgae: Underlying mechanisms and practical applications. Biotechnology Progress. 28: 299–311.

Morsy AA, Salama KHA, Kamel HA, Mansour MMF. (2012). Effect of heavy metals on plasma membrane lipids and antioxidant enzymes of *Zygophyllum* species. Eurasian Journal of Biosciences. 6:1–10. doi: 10.5053/ejobios.2012.6.0.1

Nascimento IA, Marques SSI, Cabanelas ITD, Pereira SA, Druzian JI, de Souza CO, Vich DV de Carvalho, Nascimento GC, Maurício A. (2013). Screening Microalgae Strains for Biodiesel Production: Lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. BioEnergy Research. 6:1-13. doi: 10.1007/s12155-012-9222-2.

Oliveira DT de, Turbay Vasconcelos C, Feito-

sa AMT, Aboim JB, Oliveira AN, Xavier LP, Santos AS, Gonçalves E, Nascimento L. (2018). Lipid profile analysis of three new Amazonian cyanobacteria as potential sources of biodiesel. Fuel. 234:785–788. doi: 10.1016/j.fuel.2018.07.080.

Pham TL, Dao TS, Bui HN, Pham TKN, Ngo TTH, Bui HM. (2020). Lipid production combined with removal and bioaccumulation of Pb by *Scenedesmus* sp. Green Alga. Polish Journal of Environmental Studies. 29:1785–1791. doi: 10.15244/pjoes/109277.

Post-Beittenmiller D, Roughan G, Ohlrogge JB. (1992). Regulation of plant fatty acid biosynthesis: Analysis of acyl-coenzyme a and acyl-acyl carrier protein substrate pools in spinach and pea chloroplasts. Plant Physiology. 100:923–930. doi: 10.1104/pp.100.2.923.

Rocha DN, Martins MA, Soares J, Vaz MGMV, de Oliveira Leite M, Covell L, Mendes LBB. (2019). Combination of trace elements and salt stress in different cultivation modes improves the lipid productivity of *Scenedesmus* spp. Bioresource Technology. 289: 121644. doi: 10.1016/j.biortech.2019.121644.

Sharma KK, Schuhmann H, Schenk PM. (2012). High lipid induction in microalgae for biodiesel production. Energies. 5:1532–1553. doi: 10.3390/en5051532.

Sibi G, Anuraag TS, Bafila G. (2014). Copper stress on cellular contents and fatty acid profiles in *Chlorella* species. OnLine Journal of Biological Sciences. 14:209–217. doi: 10.3844/ojbsci.2014.209.217.

Sierra-Cantor JF and Guerrero-Fajardo CA. (2017). Methods for improving the cold flow properties of biodiesel with high saturated fatty acids content: A review. Renewable and Sustainable Energy Reviews. 72:774-790.

Sinetova MA, Červený J, Zavřel T, Nedbal L. (2012). On the dynamics and constraints of batch culture growth of the cyanobacterium *Cyanothece* sp. ATCC 51142. Journal of Biotechnology. 162:148-155. doi: 10.1016/j.jbiotec.2012.04.009.

Singh JS, Kumar A, Singh M. (2019). Cyanobacteria: A sustainable and commercial bio-resource in production of bio-fertilizer and bio-fuel from waste waters. Environmental and Sustainability Indicators. 3–4:1-8. https://doi.org/10.1016/j.indic.2019.100008.

Sorate KA and Bhale P V. (2015). Biodiesel properties and automotive system compatibility issues. Renewable and Sustainable Energy Reviews. 41:777-798. doi: 10.1016/j.rser.2014.08.079.

Stanier RY, Deruelles J, Rippka R, Herdman M, Waterbury J. (1979). Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. Semantic Scholar. Microbiology. 111:1-61. doi:10.1099/00221287-111-1-1.

Sui N, Li M, Li K, Song J, Wang BS. (2010). Increase in unsaturated fatty acids in membrane lipids of *Suaeda salsa* L. enhances protection of photosystem II under high salinity. Photosynthetica. 48:623–629. doi: 10.1007/s11099-010-0080-x

Sun J, Cheng J, Yang Z, Li K, Zhou J, Cen K. (2015). Microstructures and functional groups of *Nannochloropsis* sp. cells with arsenic adsorption and lipid accumulation. Bioresource Technology. 194:305–311. doi: 10.1016/j.biortech.2015.07.041

Sunda WG, Price NM, Morel FMM. (2005). Trace Metal Ion Buffers and Their Use in Culture Studies. In: Andersen R A (ed) Algal Culturing Techniques. Elsevier Academic Press. San Diego, CA, USA. pp: 35-63.

Sytar O, Kumar A, Latowski D, Kuczynska P, Strzałka K, Prasad MNV. (2013) .Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. Acta Physiologiae Plantarum. 35:985-999

Szekeres E, Sicora C, Dragoş N, Drugă B. (2014). Selection of proper reference genes for the cyanobacterium *Synechococcus* PCC 7002 using real-time quantitative PCR. FEMS Microbiology Letters. 359:102–109. doi: 10.1111/1574-6968.12574.

Talebi AF, Mohtashami SK, Tabatabaei M, Tohidfar M, Bagheri A, Zeinalabedini M, Hadavand Mirzaei H, Mirzajanzadeh M, Malekzadeh Shafaroudi S, Bakhtiari S. (2013). Fatty acids profiling: A selective criterion for screening microalgae strains for biodiesel production. Algal Research. 2:258-267. doi: 10.1016/j.algal.2013.04.003.

Talebi AF, Tohidfar M, Bagheri A, Lyon SR, Salehi-Ashtiani K, Tabatabaei M. (2014). Manipulation of carbon flux into fatty acid biosynthesis pathway in *Dunaliella salina* using *Acc*D and ME genes to enhance lipid content and to improve produced biodiesel quality. Biofuel Research Journal. 1:91-97. doi: 10.18331/BRJ2015.1.3.6.

Tiwari ON, Bhunia B, Muthuraj M, Kanti Bandyopadhyay T, Ghosh D, Gopikrishna K. (2020). Optimization of process parameters on lipid biosynthesis for sustainable biodiesel production and evaluation of its fuel characteristics. Fuel. 269:117471. doi: 10.1016/j.fuel.2020.117471.

UNESCO. (2019). UN World Water Development Report. UN-Water. doi: https://unesdoc.unesco.org/ark:/48223/pf0000367306.

Upchurch RG. (2008) Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnology Letters. 30:967-977.

Yilancioglu K, Cokol M, Pastirmaci I, Erman B, Cetiner S. (2014). Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS One. 9:1–13. doi: 10.1371/journal.pone.0091957.

Extraction and Determination of Astaxanthin Pigment From *Haematococcus pluvialis* Microalgae

Maryam Ameri, Zahra Valiollahi, Mohsen Seyedabadi, Reza Karazhyan, Najme Gord Noshari, Parirokh Lavaee*

Received: 2021-09-15 Revised and accepted: 2021-11-24

Abstract

Astaxanthin and β-Carotene are well-known carotenoids globally, covering more than half of the market demand for carotenoids. *Haematococcus pluvialis* microalgae are one of the most important sources of natural astaxanthin, consisting of up to 4% of its dry weight. The most critical challenge for this microalgae is the breakdown of the wall and the extraction of the pigment. In this study, chemical methods, including acid, acetone, and ionic solution, and physical processes such as ultrasound waves and magnetic stirrer, were used to break down the cell wall and meas-ure total astaxanthin in *H. pluvialis*, respectively. Due to the rapid oxidation of the pigment, in the next step, to extract and store astaxanthin from damaged cells, use olive oil. A spectrophotometer examined astaxanthin, monoester, and diester derivatives, and their amount was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The results showed that using acid treatment, ultrasound waves, and extraction by acetone is the best method to measure the amount of astaxanthin in the algae. The HPLC results also showed that the amount of astaxanthin monoester (88.44%) was higher than the free forms (3.76%) and diester (7.82%) in the total content of extracted astaxanthin. In addition, the amount of total astaxanthin in the *H. pluvialis* was about 1.6% of the dry weight of the algae.

Keywords: *H. pluvialis*, Astaxanthin, Cell Disruption, Microalgae, Ultrasound Waves

Introduction

Microalgae are microorganisms that can grow in both freshwater and saltwater. Already, research on microalgae was related to the production of biofuels. Still, now they are used in the large scale production of unique and more valuable materials in large quantities, which can be helpful in the Pharmaceutical, Food, and Cosmetics Industries (Borowitzka, 2013; Koller et al., 2014; Panis and Carreon, 2016). Astaxanthin and β-Carotene are well known carotenoids globally, covering more than half of the market demand for carotenoids. Microalgae can produce valuable bio-

Industrial Microbial Biotechnology Department, Research Institute for Industrial Biotechnology, Academic Center for Education, Culture, and Research (ACECR), Mashhad, Iran.
*Email address: parilavaee@yahoo.com

chem-icals, including carotenoids, and act as a natural pool for making these substances (Suseela and Toppo, 2006). Astaxanthin is one of the most valuable carotenoids, and its global market is expected to reach 3.5 billion$ by 2026 (Basiony et al., 2022). The oxidative capacity of astaxanthin is ten times higher than other carotenoids. So, the antioxidant properties of astaxanthin make it a suitable candidate for application in aquaculture and food industries. (Reyes et al., 2014). *H. pluvialis* is the richest source of astaxanthin (1.5-4.5% dry weight of the algae) (Machmudah et al., 2006). Noroozi et al. (2012) showed two steps of cultivation are needed to produce astaxanthin. The first step is related to the green phase (bio-mass production), and the second step is associated with the red phase (astaxanthin production) (Noroozi et al., 2012). The algae cyst stored astaxanthin in a thick, rigid cell wall (Keykha akhar et al., 2021). Different methods are used to destroy the cyst cell wall to extract astaxanthin. However, the temperature and chemical stresses caused by some physicochemical extraction methods can change the chemical structure of astaxanthin, destroy astaxanthin isomers, and directly affect the quality and value of astaxanthin. The chemical composition and nature of microalgae cell walls are essential factors that should be considered to select the appropriate destruction method for recovering valuable intracellular products such as carotenoids (Cheng et al., 2015). Methods of cell wall destruction are divided into four groups: chemical, physical, physicochemical, and biological processes (Kim et al., 2016). This study extracted astaxanthin pigment from *H.pluvialis* microalgae native to Iran. After determining astaxanthin percentages in algae, also measured the amount of the oil phase (Li et al., 2012; Kim et al., 2016; Kang and Sim, 2008; Liu et al., 2018).

Material and methods

H. pluvialis microalgae powder in the red phase was prepared from Qeshm Microalgae Biorefinery. Compounds included standard Astaxanthin and 3-methyl-3-butyl imidazolium chloride (Sigma Com.) as an ionic solution, hydrochloric acid, acetone, hexane, methanol, ethyl acetate, dimethyl sulfoxide (DMSO), and Merck TLC 60 (from TLC Silica gel 60 F from MERCK), olive oil, and devices included ultrasonic cleaner bath SB-103 D (made in Korea), hot water bath (bain-marie), GFL - Gesellschaft für Labortechnik (made in Germany), HERMLE Labortechnik centrifuge (made in Germany), the HPLC, Knauer Series Wellchrom solvent organizer (K-1500) (made in Germany), and the Bio-Tek microplate reader Model Epoch (made in the USA).

Determination of astaxanthin percentage

Extraction methods with ionic solution, acid, acetone, and combination were used to determine the percentage of astaxanthin in *H. pluvialis*.

Ionic solution

Recently, ionic solutions have been used for the green extraction of carotenoids, which

methyl-3-butyl imidazolium chloride is one of the best ionic solutions with the highest efficiency (Saini and Keum, 2018, Kim et al., 2016, Desai et al., 2016) First, 10 mg of *H. pluvialis* powder was transferred to two Falcon tubes, then, 1 ml of 40% and 60% ionic solutions were added to it, and then it was placed in an ultrasonic bath (one hour), after that, the ionic solution was separated by centrifugation at a rate of 12000 g (10 minutes), and then it was washed twice with water after that 4 ml of acetone were added to it, and then the solution was placed on a shaker (1 hour) and separated in acetone by centrifugation at 12000 g (10 minutes). This operation was repeated twice, and finally, the acetone was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 1). This experiment was repeated three times.

Ionic solution and ultrasound

First, 10 mg of *H. pluvialis* powder was transferred to three falcon tubes, then 1 ml of 40% ionic solution was added to it, and after that, it was placed in an ultrasonic bath (30, 60, and 120 minutes). The ionic solution was then separated, and the precipitates were washed twice with dis-tilled water. Finally, they were incubated twice (one hour), each time with 4 ml of acetone, and the acetone was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 2). This experiment was repeated three times.

Ionic/acid solution/ultrasound

First, 10 mg of *H. pluvialis* powder was added to two Falcon tubes, then one ml of 40% ionic solution was added to one pellet and placed in a 60 °C water bath (one hour). Then, 1 ml of 4 M HCl was added to the other pellet, and it was placed in an ultrasonic bath at 70 °C (30 minutes). After that, the sample was centrifuged, the acid was removed, and then it was washed, and one milliliter of 40% ionic solution was added to the pellet, and then it was placed a 60 °C water bath (one hour). After that, the sample was centrifuged, separated, and washed. Then, acetone was added twice

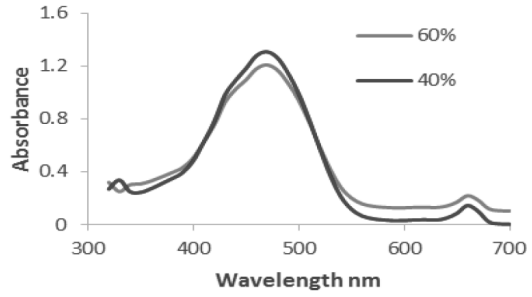

Fig. 1. UV-Vis absorption spectra of astaxanthin extracted by 40 and 60% ionic liquid

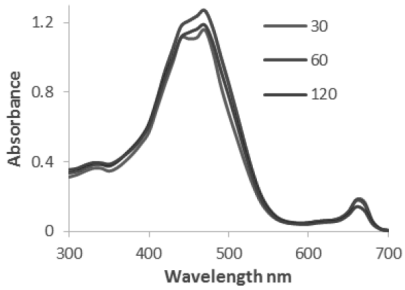

Fig. 2. UV-Vis absorption spectra of astaxanthin extracted by ionic liquid treatment and ultrasound waves at times of 30, 60 and 120 minutes

to the remaining pellets, 4 ml each time, and then it was placed in the darkness (one hour), and finally, the acetone was separated by centrifugation. It was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 3). This experiment was repeated three times.

Acid and acetone

One of the high efficiency extraction methods is to use acid to break down the cell wall of *H. pluvialis* and acetone to extract astaxanthin.

First, 10 mg of *H. pluvialis* was added to two containers. Then, 1 ml of 4 M HCl acid was added to the first container, and it was placed in a water bath (1 hour) at 70° C, and 1 ml of 4 M HCl acid was added to the second container, and it was placed in a 70° C bath on a magnetic stirrer. The acid was then removed by centrifugation, and the remaining pellets were washed with water. Acetone was added to the remaining pellets twice, 4 ml each time, and then it was placed in the darkness (one hour), and finally, the acetone was separated by centrifugation. It was brought to the desired volume in a 10 ml volumetric flask, and the absorption spectrum of the solution was recorded in the range of 350 to 700 nm (Figure 4). This experiment was repeated three times.

Acid, acetone/water bath, and glass bead sterilizers

Three microtubules containing 10 mg of *H. pluvialis* and 1 ml of 4 M HCl were prepared. The first sample was placed in a water bath at 70 °C (one hour). The second sample was first rubbed with glass bead sterilizers in a mortar, and then it was placed in the water bath (one hour). The third sample was placed in an ultrasonic bath at 70 °C (half an hour), and then it was placed in a water bath (half an hour). After one hour, the supernatant of all three centrifuged samples was discarded, and the pellets were washed twice with distilled water. Acetone was added to all samples in two stages, and after separation, they were brought to the desired volume in a 10 ml

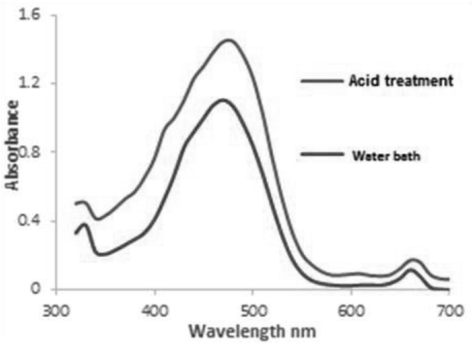

Fig. 3. UV-Vis absorption spectra of astaxanthin extracted using ionic liquid with acidic treatments and water bath

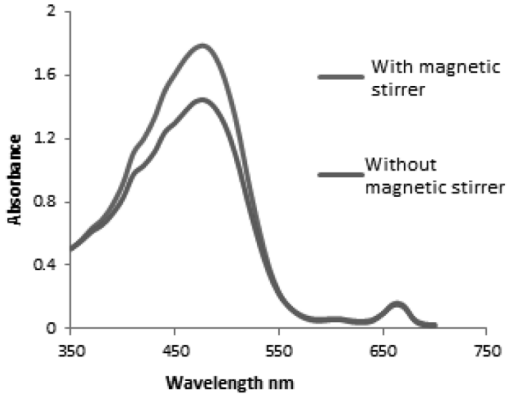

Fig. 4. UV-Vis absorption spectra of astaxanthin extracted by two methods using magnetic stirrer and without magnetic stirrer

volumetric flask. The absorption spectrum was recorded at 300 to 700 nm (Figure 5). This exper-iment was repeated three times.

Acid, acetone, and ultrasound

Two treatments (30 and 45 minutes) were examined in this test. In this test, 10 mg *H. pluvialis* powder was used for each sample. In the first case, with three replications, 1 ml of 4 M HCl was added to *H. pluvialis* powder, and then it was placed in an ultrasonic bath at 70 °C (30 minutes), and then it was placed in a water bath at 70 °C (30 minutes). In the second case, the sample was placed in an ultrasonic bath (45 minutes), and then it was placed in a water bath (15 minutes). After that, both samples were centrifuged, separated, and washed. Then acetone was added to each in two steps, and finally, it was brought to the volume of 10 ml. The absorption spectrum was recorded in the wavelength range of 300 to 700 nm (Figure 6).

Extraction of astaxanthin by olive oil with acid pretreatment and ultrasound waves

First, 100 mg of the *H. pluvialis* powder and 5 ml of 4 M HCl were added to it, and it was placed in an ultrasonic bath at 70° C (30 minutes). After completion of the ultrasound, the acid was separated from the sample by centrifugation, and then it was washed twice with distilled water. Then the remaining pellet was made up of 30 ml of distilled water, and 30 ml of olive oil was added to it and then placed on a magnetic stirrer. The absorption spectrum of the oil was recorded at a wavelength of 300 to 700 nm at 24 and 48 hours (Figure 7). After 48 hours, the sample was taken out of the water, and the sediments were separated and kept in a vial.

The extraction of olive oil with acid/bath-water pretreatment

First, 100 mg of *H. pluvialis* powder with 5 ml of 4 M HCl was placed in bath water at 70° C (one hour). After that, the acid was separated from the sample, and then it was washed with distilled water in two steps. After centrifugation, 30 ml of distilled water and 30 ml of olive oil were added to the remaining pellet, and an Erlenmeyer flask

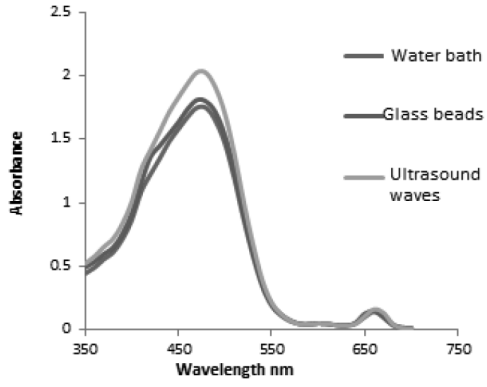

Fig. 5. UV-Vis absorption spectra of astaxanthin extracted by three methods using water bath, glass beads and ultrasound waves

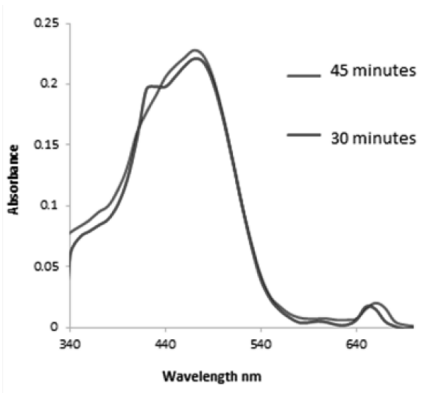

Fig. 6. UV-Vis absorption spectra of astaxanthin extracted by ultrasound pretreatment at 30 and 45 minutes

was placed on the stirrer. Then the absorption spectrum was recorded at 24 and 48 hours (Figure 8). This experiment was repeated three times.

Standard curve

To draw the standard curve of astaxanthin in acetone, first, a solution with the concentration of 1.61×10^{-4} M was prepared, and then five solutions with concentrations of 1.60×10^{-6}, 3.21×10^{-6}, 4.82×10^{-6}, 6.43×10^{-6}, and 9.65×10^{-6} were designed. In the next step, the maximum adsorption of each solution was measured with a spectrophotometer, and the adsorption curve was drawn in front of the concentration. Then the line equation was obtained, and the solutions obtained from the algae extraction were used to determine the concentration (Figure 9). To draw the standard curve of astaxanthin in olive oil, five solutions with concentrations of 8.05×10^{-7}, 1.61×10^{-6}, 4.83×10^{-6}, 9.66×10^{-6}, and 1.93×10^{-5} were also prepared. Then, their absorption spectrum was recorded at 300 to 700 nm (Figure 10).

Chromatography

Thin layer chromatography (TLC) and High performance liquid chromatography (HPLC) were used to identify and measure astaxanthin and its derivatives.

The use of thin-layer *chromatography to*

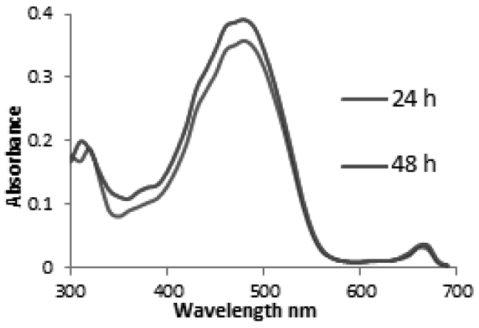

Fig. 7. UV-Vis absorption spectra of astaxanthin extracted by olive oil after 24 and 48 hours

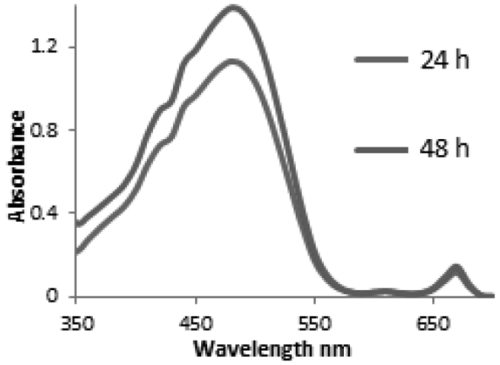

Fig. 8. UV-Vis absorption spectra of astaxanthin extracted by olive oil with acid pretreatment / water bath

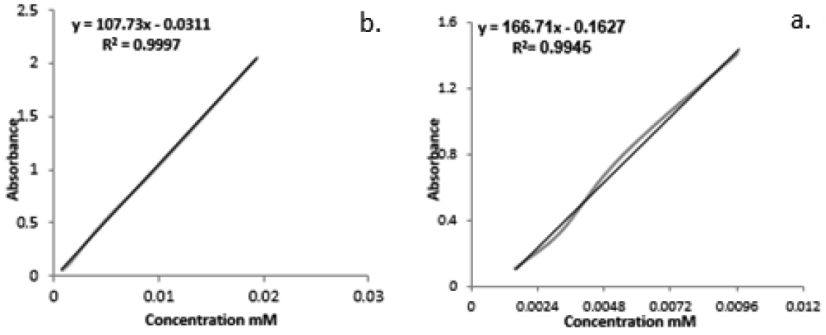

Fig. 9. Standard curve for astaxanthin in a) acetone and b) olive oil

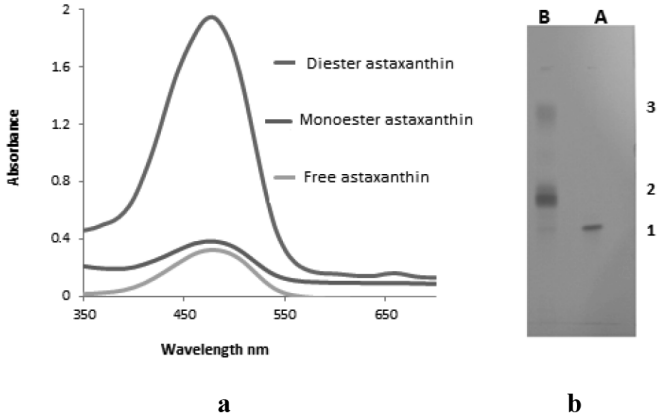

Fig. 10. a) Isolation of astaxanthin forms by TLC: A sample of standard astaxanthin, B substance extracted from *H. pulvialis,* **b**) Absorption spectrum of astaxanthin and monoester and diester derivatives isolated by TLC

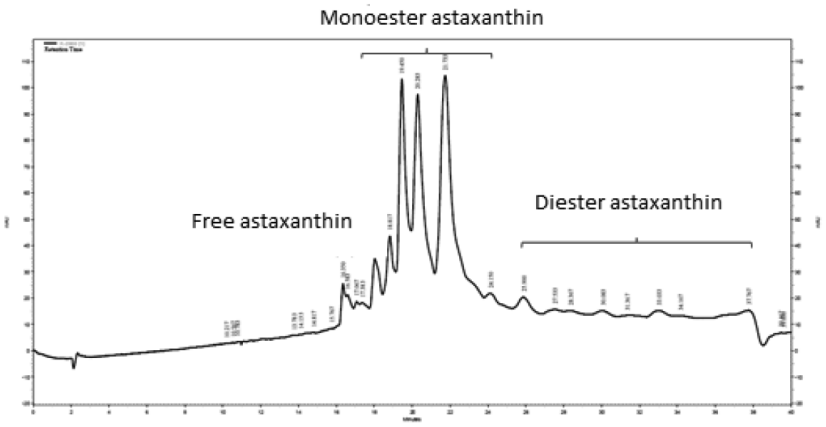

Fig. 11. Chromatogram of the extract was determined by acid-ultrasound-acetone method, which showed peaks related to free astaxanthin, monoester astaxanthin and diester astaxanthin

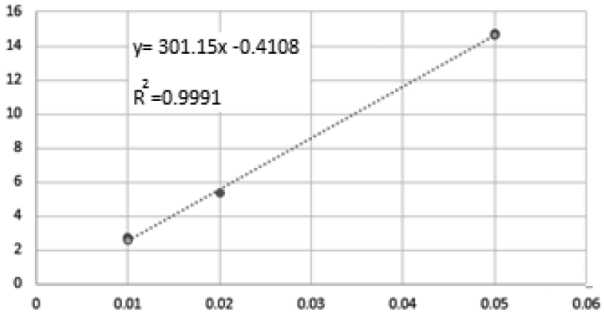

Fig. 12. Standard curve for astaxanthin by HPLC

evaluate astaxanthin extracted from H. pluvialis

The acetonic extract of *H. pluvialis* was used for further analysis, and the presence of astaxanthin and its monoester and diester derivatives was investigated by thin layer chromatography. TLC test was performed using thin layer chromatography paper (TLC Silica gel 60 F from MERCK) in a special tank in a closed environment with hexane/acetone mobile phase in the ratio of 7:3 (Orona-Navar et al., 2017) (Figure 10-a). The bands associated with free astaxanthin, astaxanthin monoester, and astaxanthin diester were isolated from TLC and extracted from silica by acetone. The absorption spectra were recorded at 350 to 700 nm (Figure 10b).

The use of high-performance liquid chromatography to evaluate astaxanthin extracted from H. pluvialis

Astaxanthin and its derivatives were investigated in the extract from *H.pluvialis* by HPLC from Knauer Well Chrom Solvent Organizer (k-1500), with pump K-1001 and UV Detector K-2600 using column C18 (10×4.6 mm) at ambient temperature, and the wavelength of 480 nm, and the flow rate of 0.8 ml/min. Run time was 40 minutes, and the mobile phase was a combination of water, and methanol, which in the first 5 minutes, isocratic with the ratio of 50:50, to 35 minutes, the ratio of two solvents as a gradient changed from 20:80 to a percentage of 0:100, and in the final 5 minutes the static phase ratio remained 50:50. Several different peaks are observed, indi-cating various derivatives of astaxanthin.

10 mg extract of *H. pluvialis* was prepared by acid ultrasound and acetone to evaluate the contribution of each of the astaxanthin derivatives and measured by chromatography (HPLC). The chromatogram spectrum of the total extract is shown in Figure (11). Free astaxanthin was inject-ed into the system to determine the peaks related to free astaxanthin, standard, and according to the exit point of the sample, the peak of free astaxanthin was identified in the chromatogram of the total extract. The whole extract was first separated by TLC to identify astaxanthin derivatives, and the bands related to the monoester and diester derivatives were separated and sepa-rately injected into the HPLC. Their peaks on the main chromatogram were determined. The relevant software calculated the area below the peaks. Then, standard concentrations of pure astaxanthin were prepared, and a standard diagram was drawn (Figure 12). The percentage of free astaxanthin and its derivatives was calculated in the sample of *H. pluvialis* by the standard diagram line equation and the area below the peaks of the tested sample and the average molar mass of the monoester and diester derivatives.

Results

Investigation of ionic solution

The results of using ionic solutions with 40% and 60% concentrations are listed in Table (1). As seen, the ionic solution with a concentration of 40% was more efficient.

According to the results for time treatments in ionic solution (Table 2), no significant difference was observed between 60, and 120 times in compare to difference between 30 and 60 minutes. So, 60 minutes treatment was considered as the best.

Determination of astaxanthin using ionic/acid solution/ultrasound

According to the results of Table (3), it can be seen that the acid/ultrasound pretreatment was more efficient.

The use of magnets for astaxanthin extraction

In this method, small magnets were used to stir the sample in the microtube. According to Figure (4), after a few repetitions, the extraction results were better when a water bath was used alone than the solution was stirred.

The Comparison of the water bath, glass bead, and ultrasound waves

In this experiment, some methods were used that are suitable for increasing the extraction per-centage, such as ultrasound waves and rubbing with glass bead sterilizers. The results (Figure 5) indicated that using a combination of ultrasound waves and a water bath showed better results than the other two methods.

Extraction of astaxanthin by acid, ultrasound, and acetone

The water bath/ultrasound method was used in 30, and 45-minute intervals and a small percent-age of the extracted astaxanthin was calculated by the standard diagram line equation (Table 4).

Extraction of astaxanthin by oil with acid pretreatment and ultrasound waves

According to Figure (7), there is no significant difference between 24 and 48 hours, and the percentage of astaxanthin was calculated to be about 1.2%.

Treatment with acid and olive oil

In this experiment, olive oil was added to

Table 1. Percentage of astaxanthin extracted with 40 and 60% ionic liquids

Extraction	Pretreatment
0.366%	40% ionic liquid
0.315%	60% ionic liquid

Table 2. Percentage of astaxanthin extracted by 40% ionic liquids at 30, 60 and 120 minutes

Treatment	Extraction
30 minutes	0.42 %
60 minutes	0.48 %
120 minutes	0.50 %

Table 3. Percentage of astaxanthin extraction using ionic liquids / acid / ultrasound

Pretreatment	Extraction
Acid/ water bath	1.00 %
Acid/ ultrasound	1.12 %

Table 4. Percentage of astaxanthin extraction with ultrasound and acetone pretreatment

Pretreatment	Extraction
Water bath/ 30 minutes ultrasound	1.321 %
Water bath/ 45 minutes ultrasound	1.328 %

the mixture in three steps, and with acid treatment, the extracted volume was estimated at 1% (Figure 8).

y=27.932x-0.1627 (Eq. 1)

Thin-layer chromatography (TLC)

As seen in Figure (11), different color bands are seen on chromatography paper related to carote-noids and chlorophyll (Elumalai et al., 2014; Orona-Navar et al., 2017). Astaxanthin with three structures, including free astaxanthin, astaxanthin monoester, and astaxanthin diester, is present in the algae extract. Given the degree of polarity of the solvent and the degree of polarity of the compounds, the astaxanthin diester moves faster on paper, followed by the astaxanthin monoes-ter, and finally, free astaxanthin (which is more polar than the other two compounds). The R_f of the three structures of free astaxanthin, astaxanthin monoester, and astaxanthin diester for *H. pluvialis*is 0.386, 0.502, and 0.817, respectively. As seen in Figure (10-b), the absorption spectra obtained from the two isolated bands of astaxanthin monoester and astaxanthin diester are similar to the absorption spectrum of astaxanthin and overlap entirely with it.

The use of high-performance liquid chromatography (HPLC)

In UV-Vis spectroscopy, as the standard di-

Table 5. The amount of free astaxanthin, monoster and diester in total extract of *H. pluvialis*

Astaxanthin forms	Free	Monoster	Diester
Peak area	0.17	3.99	0.35
Percentages of Astaxanthin forms	3.76	88.42	7.82

agram of the standard material is required to be drawn, and the absorption spectra of the three forms of astaxanthin overlap (Figure 12), the measurement is done based on the molar mass of free astaxanthin. Therefore, given the higher molar mass of mono-and diester forms, the results do not show the actual weight percentage of astaxanthin in *H. pluvialis*. Thus, HPLC should be used for a more accurate determination of astaxanthin. HPLC was used to reduce the effect of this problem by determining the actual percentage of total astaxanthin. After selecting the peak of free astaxanthin, and its derivatives, the area under the curve of each of the products, was calculated. The percentage of each of them was determined in the total extract of the algae, which is according to Table (5).

Average molar mass was used to calculate the weight percentage of each astaxanthin derivative. Since the fatty acids in astaxanthin derivatives are generally $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{18:4}$, $C_{16:0}$, and $C_{16:1}$, the average molar mass of each of the mono and diester derivatives was calculated to be 849,284, and 1101,728 g/mol, respectively.

As seen in Figure (12), by drawing a standard diagram of astaxanthin concentration based on the molar mass, free astaxanthin in the extract was determined to be 1.32%, given the average molar mass of astaxanthin mono and diester derivatives. Their constituent percentages, the percentage of total astaxanthin in *H. pluvialis*, was calculated to be 1.6%.

Discussion

The microalgae cell wall composition could be changed under different environmental and nutritional conditions. The composition of the microalgae cell wall is a critical factor that should be considered for metabolites extraction (Cheng et al., 2015). Many attempts have been reported to obtain effective methods for cell wall degradation to extract valuable compounds like carotenoids from microalgae. A main part of *H. pluvialis* red biomass is related to cell wall (Shah et al., 2016Y Kim et al., 2016). So, we attempted to disrupt the cell wall and extract astaxanthin.

Chemical methods, including acid, acetone, ionic solution, and physical processes such as ultrasound waves and magnetic stirrer were used to measure astaxanthin in *H. pluvialis*. According to the results, using each of these methods alone did not have good efficiency. Our results showed that the best outcome was related to the acid treatment with 30 minutes of ultrasound waves at 70 °C.

Molino et al., 2018 reported among different solvents (acid and base, organic and ionic solvents, etc.) without using physical methods and with one extraction step, Chloroform /methanol (1: 1 ratio) had the highest extraction percentage. However, in the case of physical and chemical pretreatment, Aceton acts more effectively (Molino et al., 2018). A similar result was observed after chemical and biological pretreatment in our experiment (Mendes-Pinto et al., 2001). Also, based on the results of using an

ionic solution, 40% showed better efficiency. Still, due to the significant difference in the results of using ultrasound waves in the extraction period of 60 minutes, it is significantly different from the time of 30 minutes, while there is no significant difference with the time of 120 minutes. We obtained 60 minutes treatment as the best for astaxanthin extraction, as Choi et al. (2019) reported. Also, Ruenngam et al. (2010) and Molino et al. (2018) mentioned the negative effect of time elongation on astaxanthin extraction. Choi et al. (2019) showed high extraction by ionic solution while we didn't get a good efficiency by ionic solution. So a combination of ionic solution with acid and ultrasound wave is proposed for astaxanthin extraction. The use of 40% ionic solution, contrary to what is stated in some sources, did not have good re-sults alone, and acid treatment was used to improve the results. Due to the rapid oxidation of the pigment, olive oil was used to extract and store astaxanthin from damaged cells. According to the results, acid treatment and then extraction by olive oil led to the best yield of about 95% of the total astaxanthin in *H. pluvialis*. A spectrophotometer examined astaxanthin, monoester, and diester derivatives, and their amount was determined by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). The results showed that the best method to measure the amount of astaxanthin in *H. pluvialis* is acid treatment, ultrasound waves, and extraction by acetone. In addition, the results of HPLC showed that the amount of astaxanthin monoester (88.44%) was higher than the free forms (3.76%) and diester (7.82%) in the total content of extracted astaxanthin. The amount of total astaxanthin in the studied *H. pluvialis* was calculated to be about 1.6% of the dry weight of the algae.

Acknowledgment

Authors would like to express our special thanks to Academic Center for Education, Culture, and Research (ACECR), Mashhad for financially supporting this research.

References

Basiony M, Ouyang L, Wang D, Yu J, Zhou L, Zhu M, Wang X, Feng J, Dai J, Shen Y. (2022). Optimization of microbial cell factories for astaxanthin production: Biosynthesis and regulations, engineering strategies and fermentation optimization strategies. Synthetic and Systems Biotechnology. 7: 689-704.

Borowitzka MA. (2013). High-value products from microalgae—their development and commercialisation. Journal of Applied Phycology. 25: 743-756.

Cheng YS, Labavitch J, Vandergheynst J. (2015). Elevated CO_2 concentration impacts cell wall polysaccharide composition of green microalgae of the genus *Chlorella*. Letters in Applied Microbiology. 60: 1-7.

Choi SA, Oh YK, Lee J, Sim SJ, Hong ME, Park JY, Kim MS, Kim SW, Lee JS. (2019). High efficiency cell disruption and astaxanthin recovery from *Haematococcus pluvialis*

cyst cells using room temperature imidazolium-based ionic liquid/water mixtures. Bioresource Technology. 274: 120-126.

Desai RK, Streefland M, Wijffels RH, EppinK MH. (2016). Novel astaxanthin extraction from *Haematococcus pluvialis* using cell permeabilising ionic liquids. Green Chemistry. 18: 1261-1267.

Elumalai S, Santhose BI, Kanna GR. (2014). Extraction of carotenoid and thin layer chromatography (TLC), GC-MS, FT-IR and HPLC analysis of pharmaceutically important pigment astaxanthin from a new strain of *Haematococcus pluvialis*. Weekly Science Research Journal. 2: 2321-7871.

Kang CD and Sim SJ. (2008). Direct extraction of astaxanthin from Haematococcus culture using vegetable oils. Biotechnology Letters. 30: 441-444.

Keykha akhar F, Fakhrfeshani M, Alipour H, Ameri M. (2021). Microalgal pigments: An introduction to their biosynthesis, applications and genetic engineering. Journal of Plant Molecular Breeding.10.22058/JPMB.2021.140046.1218.

Kim DY, Vijayan D, Praveenkumar R, Han JI, Lee K, Park JY, Chang WS, Lee JS, Oh YK. (2016). Cell wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*. Bioresource Technology. 199: 300-310.

Koller M, Muhr A, Braunegg G. (2014). Microalgae as versatile cellular factories for valued products. Algal Research. 6: 52-63.

Li Y, Miao F, Geng Y, Lu D, Zhang C, Zeng M. (2012). Accurate quantification of astaxanthin from *Haematococcus* crude extract spectrophotometrically. Chinese Journal of Oceanology and Limnology. 30: 627-637.

Liu ZW, Zeng XA, Cheng JH, Liu DB, Aadil RM. (2018). The efficiency and comparison of novel techniques for cell wall disruption in astaxanthin extraction from *Haematococcus pluvialis*. International Journal of Food Science and Technology. 53: 2212-2219.

Machmudah S, Shotipruk A, Goto M, Sasaki M. Hirose T. (2006). Extraction of astaxanthin from *Haematococcus pluvialis* using supercritical CO_2 and ethanol as entrainer. Industrial and Engineering Chemistry Research. 45: 3652-3657.

Molino A, Rimauro J, Casella P, Cerbone A, Larocca V, Chianese S, Karatza D, Mehariya S, Ferraro A, Hristoforou E. (2018). Extraction of astaxanthin from microalga *Haematococcus pluvialis* in red phase by using generally recognized as safe solvents and accelerated extraction. Journal of Biotechnology. 283: 51-61.

Noroozi M, Omar H, Napis S, Hejazi MA, Tan SG. (2012). Comparative biodiversity and effect of different media on growth and astaxanthin content of nine geographical strains of *Haematococcus pluvialis*. African Journal of Biotechnology. 11: 15049-15059.

Orona-Navar A, Aguilar-Hernández I, Cerdán-Pasarán A, López-Luke T, Rodríguez-Delgado M, Cárdenas-Chávez D, Cepeda-Pérez E, Ornelas-Soto N. (2017). Astaxanthin from *Haematococcus pluvialis* as a natural photosensitizer for dye sensitized solar cell. Algal Research. 26: 15-24.

Panis G and Carreon JR. (2016). Commercial astaxanthin production derived by green

alga *Haematococcus pluvialis*: A microalgae process model and a techno-economic assessment all through production line. Algal Research. 18: 175-190.

Panis G and Carreon JR. (2016). Commercial astaxanthin production derived by green alga *Haematococcus pluvialis*: A microalgae process model and a techno economic assessment all through production line. Algal Research. 18: 175-190.

Reyes FA, Mendiola JA, Ibanez E, Del Valle JM. (2014). Astaxanthin extraction from *Haematococcus pluvialis* using CO_2-expanded ethanol. The Journal of Supercritical Fluids. 92: 75-83.

Saini RK and Keum YS. (2018). Carotenoid extraction methods: A review of recent developments. Food chemistry. 240: 90-103.

Shah M, Mahfuzur R, Liang Y, Cheng JJ, Daroch M. (2016). Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. Frontiers in Plant Science. 7: 531.

Suseela M and Toppo K. (2006). *Haematococcus pluvialis*–A green alga, richest natural source of astaxanthin. Current Science. 90: 1602-1603.