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

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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

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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

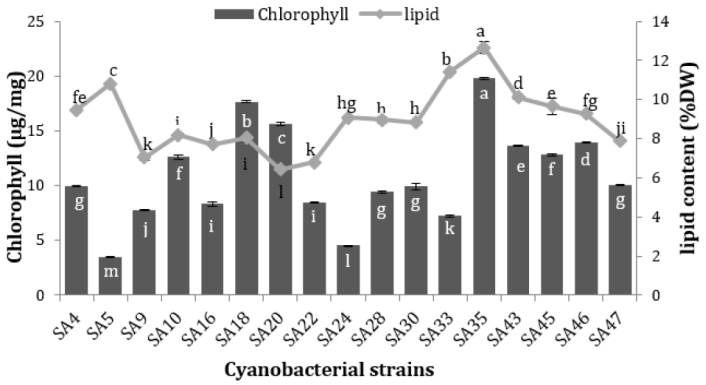

Fig. 1. Chl a and lipid content of cyanobacterial strains measured after 21st day of cultivation.

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

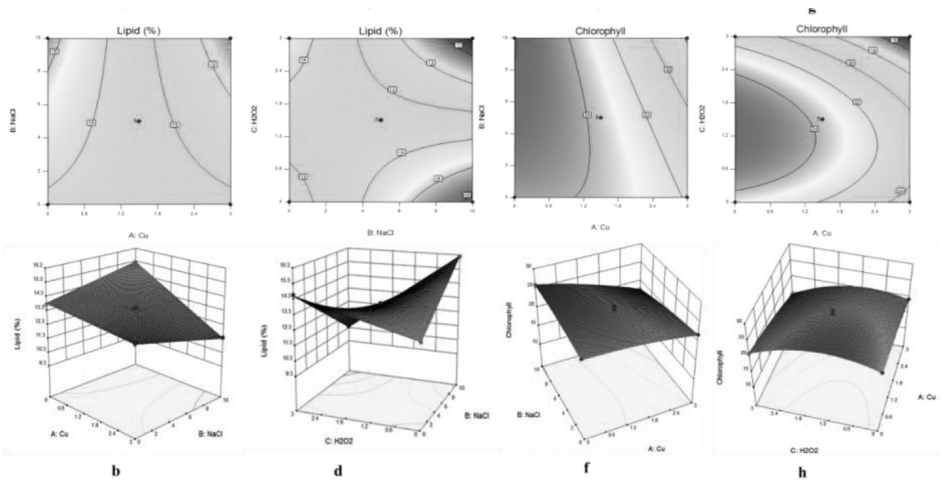

Fig. 2. Three-dimensional response plots and two-dimensional contour plots for lipid (%DW) and chlorophyll content (µg/mg). a,b) interactions of A ($CuSO_4$ µM) and B (NaClmM) and c,d) interaction of C (H_2O_2 µM) and B (NaCl mM) on lipid content enhancement. e,f) interactions of A ($CuSO_4$ µM) and 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

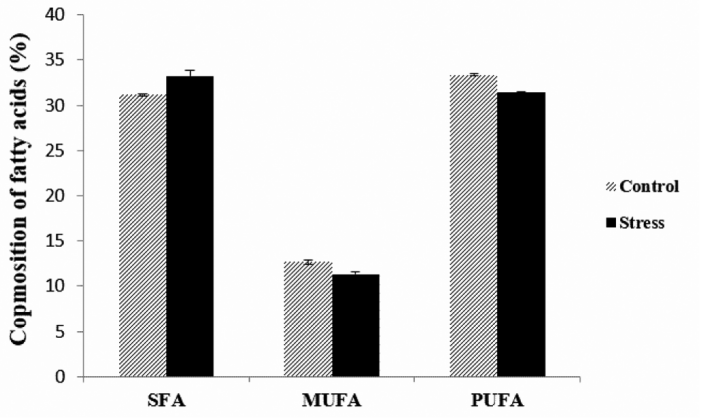

Fig. 3. Percentage contribution comparison of SFA, MUFA, PUFA of *Aliinostoc* sp. SA35 strain in control ($BG\text{-}11_0$) and POC (10 mM NaCl, 3 µM $CuSO_4$ and 0 µM H_2O_2)

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

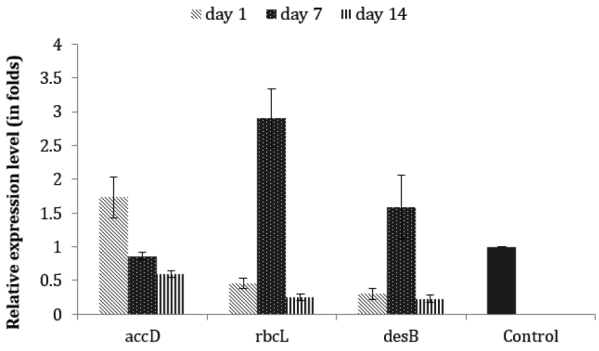

Fig. 4. Expression of *acc*D, *rbc*L and *des*B genes in *Aliinostoc* sp. SA35 strain exposed to POC treatment for 1, 7 and 14 days. Values were normalized to levels of *sec*A, a housekeeping gene

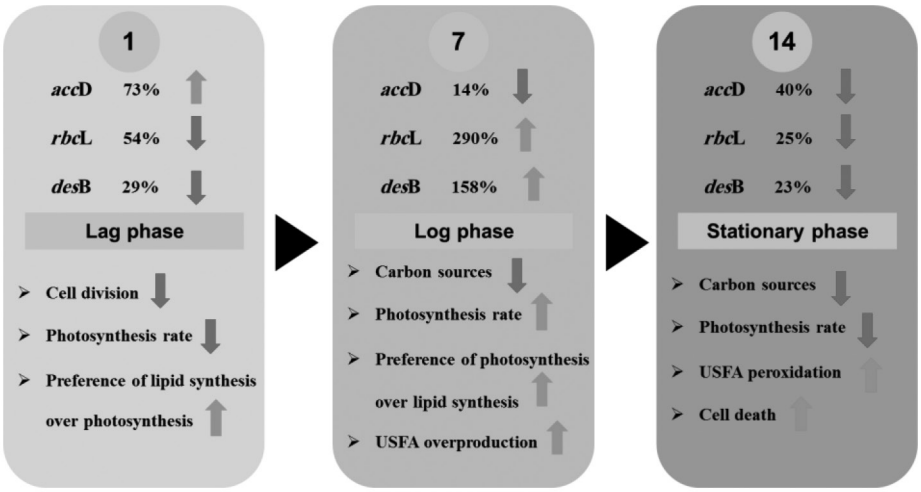

Fig. 5. The *acc*D, *rbc*L and *des*B genes expression variation under POC during two weeks (dayc1, 7 and 14). Blue arrows: up-regulation; Red arrows: down-regulation. (Expression percentage is calculated compared to the control)

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).

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