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Gene expression of *Acetyl Co-A carboxylase A* (*accA*) in *Synechocystis salina* under nitrogen starvation and low salinity stress

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Abstract: Cyanobacteria are potential sources of valuable products such as proteins, lipids, pigments, vitamins and polysaccharides. lipids are considered one of the primary active metabolites in the cyanobacterium cell. Therefore, the usage of the unicellular cyanobacterium *Synechocystis salina* as a sustainable biological source for the accumulation of lipids is the main goal of the current research. Environmental stress such as low salinity (25 ppt sea salt) increased the lipid content of *S.salina* to 13.883% DW compared with the control. Nitrogen deficiency also elevated the lipid content of *S.salina* to 11.87% DW compared with control at adequate N supply. Acetyl Co-A carboxylase A (*accA*) catalyzes the first step in fatty acid biosynthesis; therefore, the monitoring of its transcription level is crucial to understand the effect of different stressors on lipid biosynthesis. The expression level of *accA* gene in *S.salina* was upregulated to 8.38 and 35 folds under nitrate depletion and 25 ppt sea salt respectively.

keywords: Gene expression, *Acetyl Co-A carboxylase A* (*accA*), low-salt stress, nitrogen starvation

Introduction

Cyanobacteria are photosynthetic microorganisms that inhabit different environments including moist soil, bare rocks, oceans, fresh and brackish water. These blue-green microorganisms are considered as the first oxygenic photosynthetic microorganisms on the earth [1]. Cyanobacteria can grow rapidly adapting to extreme environmental changes [2]. They can produce high nutritional value products such as carotenoids, phycobiliproteins and PUFAs to different extents according to culturing conditions [3, 4]. Salinity and nitrogen starvation are critical abiotic stresses affecting microalgal growth and lipid metabolism [5, 6]. The capacity of the microalgae for adapting to salinity stress differs from one strain to another. Marine microalgae can tolerate the variation in salt concentration to a great extent compared to freshwater microalgae [7]. The effect of nitrogen depletion and salt stress on lipids accumulation was studied in *Dunaliella salina* as a valuable unicellular marine microalgae for understanding the adaptation mechanisms to abiotic stress [8]**.** Lipids are one of the primary metabolites produced in the lag and log phases of the microalgal growth curve [9]. The accumulation of polar lipids in the microalgal cell is considered a rapid response to environmental stress [10]. The metabolic pathway of fatty acid synthesis begins with the formation of malonyl Co-A after ATP-dependent carboxylation of acetyl Co A that is catalyzed by the action of acetyl Co-A carboxylase (*ACCase*) [11]. *ACCase* is recognized as the key regulator of lipid biosynthesis with four main subunits encoded for the heteromeric *ACCase* that are *accA*, *accB*, *accC* and *accD* [12]. The *accA* is the gene responsible for encoding of the alpha subunit of carboxyltransferase (α-CT) [11], while, the *accB* is encoding for biotin -carboxyl carrier protein, biotin carboxylase is encoded by *accC* and the beta subunit of carboxyltransferase (β-CT) is encoded by *accD* [12]. The microalgal ability to flourish under the influence of salt and osmotic stresses has received considerable attention; therefore, the

current study focuses on the determination of expression level of *accA* gene under two different stressors nitrate depletion and salt stress in the unicellular blue-green cyanobacterium *Synechocystis salina* with the help of quantitative PCR. Realtime PCR is an innovating tool in molecular biology as it speeds up the rate of progress in studying genes and genomics [13]. The qRT-PCR is one of highly sensitive, rapid and precise tool used in the amplification and detection of the amount of the amplified DNA [14]. The quantification of the amplicon is due to the presence of either fluorescent dye such as SYBER GREEN or fluorescent binding oligonucleotide probes which complement the targeted DNA and the intensity of the emitted light is directly correlated to the amount of the amplified DNA. The emitted fluorescence was measured at certain point called crossing point or cycle threshold (CT) [15].

2. Materials and methods

2.1. Cyanobacterium culturing

An purified single colony of marine cyanobacterium *Synechocystis salina* isolated from the Mediterranean Sea was cultivated in 20 mL artificial sea water- enriched f/2 medium [16] with a 35 ppt salt concentration. The incubation was carried out under the standard incubation conditions of 26°C, 20 μmol m^{-2} s^{-1} photon flux density from white fluorescent light for 12 -12 hours light/dark for 14 days. The purified culture after microscopic examination was transferred into a sterilized 100 mL bottle.

2.2. Effect of nitrate starvation and low salt stress on microalgal growth

The experiment was designed for studying the expression level of *accA* gene in *Synechocystis salina* under two different stressors that were nitrate depletion and salinity of 25 ppt sea salt.

For preparation of f/2 medium with a 35 ppt salt, 42 gm of artificial sea salt were dissolved in 1200 mL dist. water and 2400 μL of the sterilized f/2 medium were mixed well, 120 mL of *S.salina* inoculum was added, after mixing well, a homogenous distribution of the prepared culture into three 500 mL Erlenmeyer flasks was taken place. All flasks were incubated under standard incubation conditions and supplied by aeration that was continuously provided by bubbling. Nitrate was measured in the culture medium using a nitrate kit. After 5 days of incubation, the 25 ppt and control flasks were harvested by centrifugation at 4000 rpm for 10 minutes. On the other hand, the three flasks with 35 ppt remained under incubation till nitrogen depletion as a nitrate stress. The pellets of all treatments were collected for lipid estimation and RNA extraction.

2.3. Dry weight determination

By the end of incubation time for each treatment, all the volume of the algal culture was centrifuged at 4000 rpm for 10 min, the precipitated algal biomass was collected in pre-weighted falcon tubes and dried in the oven overnight at 60°C till constant weight.

2.4. Lipid extraction and estimation

The freeze-dried algal biomass was used for lipid extraction and quantification according to the protocol of Lewis et al. [17], a 0.02 gm of the freeze-dried biomass was soaked in 5mL of (chloroform : methanol 2:1v/v), the mixture was vortexed and stored in a refrigerator for exchanging the solvent every 48 hours for three times till clearness of solution. The collected solvent was centrifuged at 4000 rpm for 5 minutes for lipid estimation. For lipid estimation a rapid colorimetric method was used depending on sulfo-phospho vanillin reagent (SPV) [18]. The resulted pink color was spectrophotometrically measured at 530 nm. Different concentrations (50:500 μg/mL) were prepared for standard curve.

2.5. Gene expression analysis via qRT-PCR

Total RNA was extracted from algal samples according to acid guanidinium thiocyanate-phenol-chloroform extraction protocol or (TRIZol) developed by [19] and modified by [20]. The quality and quantity of the total RNA was determined by agarose gel electrophoresis and nanodrop spectrophotometer at 230, 260, 280 nm.

The complementary DNA strand (c-DNA) was constructed using a RT-PCR kit (cat. no. RR066A, TaKaRa Bio Inc, Ohtsu, Japan), A definite volume from the extracted RNA was mixed with random hexamer primer Thermo Scientific™ and Oligo (dT)18 Primer Thermo Scientific™ to be incubated at 65°C for 5 min followed by chilling. A master mix of 1x

reaction buffer, dNTPs, nuclease free water and MLV Reverse Transcriptase enzyme (Promega Catalog Number M1701) was prepared to be homogenously distributed for each sample. The prepared samples were amplified through conventional thermocycler with the following program: 42°C for 60 min, 85°C for 5min and holding phase at 4°C. The primers encoded for *accA* and housekeeping genes were retrieved from NCBI genome sequence data bases shown in Table 1. The quantitative-real time PCR was performed using SYBR Green master mix Catalog number: 4472908 (Applied Biosystems™), the 16s rRNA gene was used as normalizer gene for *S. salina*. All PCR reaction mixtures were performed with total volume 10 µL containing 1µL cDNA template, 5 µL of SYBR master mix, 0.7 µL of each primer. Real-time system (CFX96™, Bio-Rad, USA) was adjusted for 50 cycles of 95°C for 10 min as initial denaturation step, 95°C for 30 sec denaturation, annealing temperature was 56°C for 45 sec and 72°C for 1min as extension step.

The $2^{-\Delta\Delta Ct}$ method was used to determine the fold change of *accA* gene [21].

Table 1 Primers set used in qRT-PCR

Gene	Sequence 5′-3′
accA-F	GATCAGCGTGTTCCATCAA
accA-R	GGAAGGGATACCAAGGATA AG
16s rRNA-F	GCGGTGAAATGCGTAGAT
*16s-rRNA*R	CTTTCGCTACCCTAGCTTTC

3. Results and Discussion

Fig. 1 shows the effect of nitrate depletion and low salt stress on lipid content of *S.salina.* The depletion of nitrate from the culture medium led to a significant increment in lipid percentage 11.87% ± 0.38 above the control that was 7.566% ± 0.46.

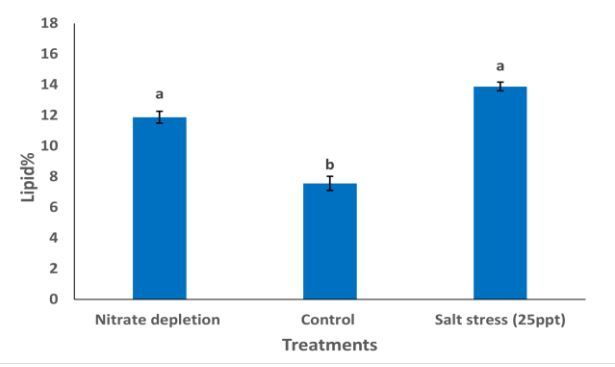

Fig. 1 Lipid content of *S.salina* under nitrate depletion and low-salt stress (25 ppt salt).

Likewise, lowering salt level of the culture medium from 35 ppt to 25 ppt led to an increase in lipid content of the cultivated *S.salina* (13.883% ± 0.283 above the control). It was even greater than that was induced by N deficiency. While nitrate deprivation led to non-significant increase in algal biomass (0.1628 ± 0.006 above the control) that was 0.1478 ± 0.007 gm **Fig. 2**, lowering salt level of the medium from 35 ppt to 25 ppt increased algal biomass significantly (0.1853 ± 0.005) comparing with control.

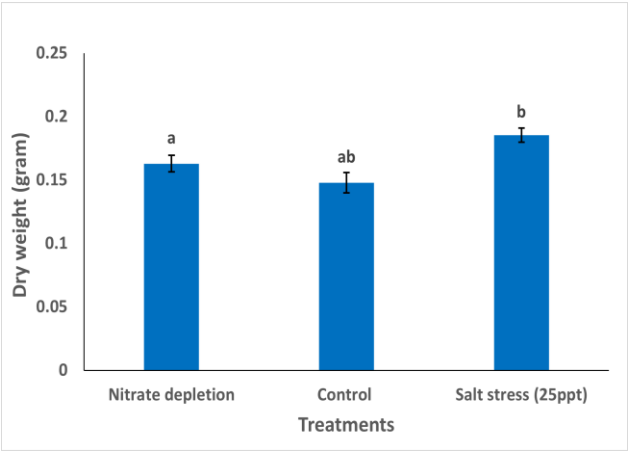

Fig. 2. Dry weight of *S.salina* under nitrate starvation and low-salt stress.

Nitrogen is considered the greatest macronutrient required by microalgal cell. It is involved in the building blocks of proteins and nucleic acids. Microalgae can grow on a medium containing nitrate or ammonium as a nitrogen source, but they can't utilize nitrite as it is considered a toxic nitrogen source. Microalgae exhibit a maximal growth rate with a nitrate as nitrogen source. If this macronutrient is depleted from the growth medium, the microalgal cells tend to shift the metabolic pathways to adapt with these conditions [22].

According to the obtained results, nitrogen starvation caused an accumulation of lipids as well as increment of the *S.salina* dry weight.

These results agree with the studies on marine microalgae [23] confirming that depletion of nitrogen from the growth medium leads to increasing in the lipid content of the alga by a 2-3 folds.

The current results agree with those of [24] declaring that the lipid content of the heterotrophic cyanobacterium *Phormidium*

autumnale increased under depletion of nitrate from the culture medium.

The capacity of the microalgae to adapt to variation in salinity level of the culture medium differs from one strain to another. Salt level significantly affects cell growth and the macromolecule composition such as proteins, lipids, and carbohydrates, it was noticed that the yield of lipids increased by lowering the salinity level of the medium. A possible explanation of this increment could be that lowering salinity affects the microalgal metabolism and this effect can be either because of the specific effects of the different ion or water osmotic potential effect that induces different osmoregulatory mechanisms within the alga to adapt to the environment [25].

As several studies indicated, the accumulation of lipids under abiotic stress is one of the main strategies for the protection of microorganisms, with enhanced desaturation of fatty acids in their membranes [26, 27].

The present results are in agreement with [28] who showed that salt stress enhanced the accumulation of total lipids in *Synechococcus* sp. *PCC 7942*.

The results agreed with [29] confirming that the lipid yield and biomass of *Microchloropsis salina* were significantly improved under salt stress.

On the other hand, the current results disagree with [30] who declared that achieving high biomass productivity and high lipid content simultanously in microalgae is diffucult. Usually the factors leading to high lipid content lead to low biomass productivity. The significant increament of the *S.salina* biomass under 25 ppt salt concentration relative to control could be due to adaptation of the marine cyanobacterium to salt flucttuation by different adaption mechanisms [31].

The current results reveal that the transcription level of *accA* gene in *Synechocystissalina* under nitrate depletion and 25 ppt low-salt stress were clearly upregulated by 8.38 and 35.41 folds respectively relative to control

Fig. 3 .

Culturing of *S.salina* on sea water based f/2 medium with salt concentration of 25 ppt led to a significant increment in the expression of *accA* while, under nitrogen depletion mode, the increase in gene expression was non-significant.

Nitrogen is considered the greatest macronutrient required by microalgal cell. It is involved in the building blocks of proteins and nucleic acids. Microalgae can grow on a medium containing nitrate or ammonium as a nitrogen source, but they can't utilize nitrite as it is considered a toxic nitrogen source. Microalgae exhibit a maximal growth rate with a nitrate as nitrogen source. If this macronutrient is depleted from the growth medium, the microalgal cells tend to shift the metabolic pathways to adapt with these conditions [22].

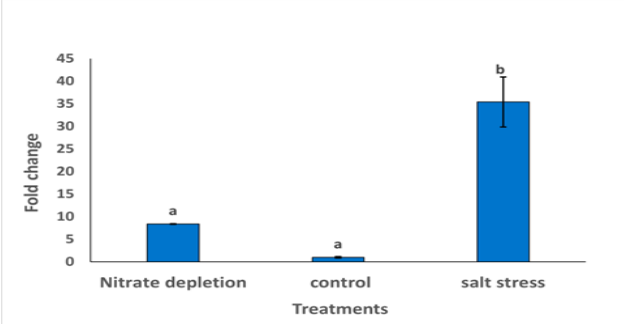

Fig. 3 Relative gene expression of *accA* in *S.salina* under nitrate depletion and low- salt stress.

The Acetyl-CoA carboxylase (ACCase) is recognized as a rate-limiting enzyme in long-chain fatty acid biosynthesis [32]. Acetyl-CoA can be catalyzed to malonyl-Co A by the action of Acetyl-CoA carboxylase which considered a crucial step in fatty acid metabolic pathway [11].

The *Accase* overall expression pattern may be controlled by the expression level of one of its subunits [33].

Environmental stresses such as nitrogen depletion and low-salt stress induce lipid accumulation in the algal cell which can be attributed to upregulation of the *accA* gene involved in the metabolic pathway of lipids [34].

These results were in an agreement with [35], who confirmed that the *accA* expression level was also upregulated under stress conditions in the mixotrophic mode of

microalgal culture.

The deficiency of nitrogen from the cyanobacterium growth medium also increased the expression level of *accA* which is in line with [36].

Lipid yield and *accA* expression level as well were enhanced in a unicellular cyanobacterium *Synechocystis* sp. PCC 6803 under abiotic stress which agreed with the current results [37].

The current results also agreed with [38] confirming that the *accA* expression level was upregulated under stress conditions in *Microcrocoleus* and *Oscilattoria* sp.

[34] concluding that there is a significant upregulation of *accA* under nitrogen deficiency in the green microalgal sp.

The present findings can be summarized as both nitrogen deficiency and low-salt stress could induce the transcriptional level and regulation of *accA* which further increases the accumulation of lipids of the marine cyanobacterium *S.salina*.

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