International Journal of Microbiological Research 10 (3): 111-114, 2019
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2019.111.114

Expression of Fatty Acid Synthesis Gene in *Chlorococcopsis minuta* under Nitrogen Deprivation

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Abstract: Nutrient depletion induces the fatty acid accumulation in several microalgae. In this study, *Chlorococcopsis minuta* was cultivated under nitrogen depleted conditions to determine the effect of nitrogen deprivation on fatty acid biosynthesis gene. cDNA synthesis and gene expression of fatty acid synthesis related gene was explored using quantitative real-time RT-PCR for the selected accD gene. Beta actin gene was used as internal control and the 2^ΔΔCt fold variation was calculated. Results showed that as compared to the control sample (0.0025), the test sample gave a fold variation of 1.000, indicating very high level of regulation of accD gene. Conclusion: The results indicated that accD gene could be a potential candidate for higher production of fatty acids for microalgae based biofuel.

Key words: *Chlorococcopsis minuta* · Fatty Acid · Nitrogen Deprivation · accD gene

INTRODUCTION

Biofuel from microalgae is mainly focused on comparing fatty acid composition in microalgae and the role of various stresses on fatty acid content and composition in microalgae [1-3]. It is well known that microalgae biofuels could not make an impact on the fuel market until they are economically feasible. One of the main barriers is the high producing cost due to our lacking of understanding of microalgal growth, metabolism and biofuels production. The fatty acid composition of microalgae is dramatically influenced by cultivation mode [4], nutrition stress [5] and environmental factors [6]. Numerous studies have focused on the impacts of nutrition stress on the fatty acid composition of microalgae, where nitrogen accounts for the majority [7, 8]. Nitrogen starvation is generally considered as the most common and effective way to trigger lipid accumulation. In recent years, more emphasis focuses on discovering metabolic engineering methods to improve content of microalgal FAs *in vivo* [9-12]. In our previous study [13], the lipid content of *Chlorococcopsis minuta* showed drastic changes during nitrogen limitation and an attempt was made in this study to understand the expression of key FA biosynthesis gene in the formation of neutral lipids. The fatty acid gene expression pattern was studied using quantitative real-time RT-PCR under nitrogen depletion conditions.

MATERIALS AND METHODS

Cultivation Conditions: *Chlorococcopsis minuta* isolated from previous study was cultivated in Bolds' basal media under controlled laboratory conditions (temperature 25°C, light intensity of 60 µmol photons m^{-2} s^{-1} and a light/dark cycle of 12/12 hours). Water losses through evaporation were maintained by adding required amount of distilled water to cultures. All experiments were carried out in triplicates for a period of 16 days. Nutrient stress was induced by avoiding the addition of any kind of nitrogen source to the media. Bold's basal media with a suitable nitrogen source was used for control.

RNA Isolation: *Chlorococcopsis minuta* cultures that had produced lipid droplets under nitrogen starvation from previous study [13] were used for RNA isolation. RNA was isolated using Standard RNAsol™ kit according to the manufacturer instructions (Chromous Biotech™).

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Table 1: Gene used in the study

Gene	Primer sequence	Amplicon size	Annealing
accD	5' – GATGCTGTTCAAACAGGAACAGGAC – 3'	198 bp	52°C
	5' – AATACCTTCTTGCATTCGAGCTCC– 3'		

Algal cells were rinsed with DEPC treated water and homogenized in a pestle and mortar with 600 µl of RNAsol™ placed on dry ice. The viscous liquid was transferred to an Eppendorf's tube and 200 µl of Chloroform: Isoamyl alcohol mixture (24:1) and mixed. The Eppendorf tube was centrifuged at 10, 000 rpm for 5 minutes at room temperature. The upper clear aqueous layer was carefully transferred into a separate fresh Eppendorf tube and equal volumes of isopropanol were added. The mixture was centrifuged at 5600 x g for 1 minute at room temperature and the procedure was repeated thrice. To the supernatant, 30 µl of warm elution buffer (~65°C) was added and incubated at 65°C for 2 minutes followed by centrifugation at 5600 xg for 1 minute at room temperature. The elution process was repeated for multiple times and the sample was collected in a fresh DEPC treated vial. Total RNA isolation was confirmed by UV Spectrophotometry.

Selection of Gene: accD gene was selected based on it's over expression under nitrogen deficient conditions in microalgae and was used for specific gene expression studies. The nucleotide sequence of the selected gene was obtained in FASTA format from NCBI database and the apt forward primer and reverse primer were selected.

cDNA Synthesis: The cDNA was synthesized using the isolated RNA as template in a q-RT PCR. The 'First Strand Synthesis kit' was used for reverse transcription process followed by the use of SYBR Green for real-time PCR (non-specific). The isolated RNA was incubated with the oligo dT primer at 65°C for 15 minutes. The RT buffer, RNasin (10U/µl), dNTPs (2.5 mM each), DTT (100 mM), MMLV RT (20 U/µl) was added and the mixture was incubated at 42°C for 1 hour. The reverse transcription reaction was inactivated by heat treatment at 94°C for 5 min. RNase was then added to the mixture to degrade the RNA strand and the only remaining ssDNA was used for the further qPCR.

PCR Experiments for Gene Amplification: PCR Conditions included Template (first strand cDNA) 2.0 µl; Forward Primer 2.0 µl; Reverse primer 2.0 µl; 10X Assay Buffer 5.0 µl; dNTPs (10mM) 2.0 µl; FasTaq (3U/µl) 0.5 µl; Water 37.5 µl; Total reaction volume 50.0 µl. The PCR conditions include 94°C for 5 min followed by 40 cycles of 94°C for 5 s, 52°C for 10 s and 72°C for 10 s and finally an extension of 1 min.

qRT-PCR: The master-mix for qPCR was added to the ssDNA along with the SYBR Green dye and gene specific primers (5' - GATGCTGTTCAAACAGGAACAGGAC - 3' and 5' - AATACCTTCTTGCATTCGAGCTCC - 3'). First strand cDNA was used for PCR and the conditions included Template (first strand cDNA) 2.0 µl; Forward Primer 2.0 µl; Reverse primer 2.0 µl; 2 X PCR SYBR green ready mix 25.0 µl; Water 20.0 µl; Total reaction volume 50.0 µl. qRT-PCR was performed using ABI Step-one Real Time PCR machine. The 2^ΔΔCt was used to calculate fold change in the expression of genes under the above-specified conditions. Beta actin gene was used as internal control.

RESULTS

Chlorococcopsis minuta cells grown under nitrogen deprived conditions were harvested for RNA isolation. cDNA synthesis and gene expression of fatty acid synthesis related gene was explored using quantitative real-time RT-PCR. The qRT-PCR gene level expression analysis was performed in comparison to given control sample. A 2^ΔΔCt fold variation was calculated. As compared to the control sample (0.0025), the test sample gave a fold variation of 1.000, indicating very high level of regulation (Table 2).

In our previous study, combinations of N deplete and replete conditions were used to generate genotypic responses with respect to lipid droplet accumulation in *C. minuta*. Gene expression studies were performed to understand the response of essential genes involved in de novo fatty acid (FA) biosynthesis and triacylglycerol (TAG) production under the same conditions.

Although recent transcriptomic and modelling studies to determine the lipid metabolism in microalgae have thrown light on diversion of lipid metabolism, there is no clear indication on the determining factors behind lipid droplet in *C. minuta* under N sufficient or limited condition. In fact, accD gene is up-regulated under N deficient conditions. The up-regulation of accD also strongly correlates with lipid content. The study provides

Table 2: Relative gene expression analysis

Sample	Mean Ct value	Δ Ct	ΔΔ Ct	2^ΔΔCt
Control	33.9285	6.9437	-8.6438	0.0025
Nutrient deprivation	21.7277	-1.7001	0.0000	1.0000

Ct-1, Ct-2, Ct-3 are Ct values from 3 independent qRT-PCR reactions

Δ Ct is the difference between mean Ct of gene (accD) and mean Ct of actin (housekeeping gene)

ΔΔ Ct is the difference between the mean Ct of Control sample and the test sample, for accD gene

2ΔΔCt is the representation of Fold variation in level of expression of accD between the test sample and control sample

us with a comprehensive picture of targeted gene that can be used for systematic metabolic engineering. Furthermore, the results also represent the feasibility of lipid production through cultivation-modes, among which nitrogen deprivation is suggested as the best way for lipid production method in *C. minuta*.

DISCUSSION

To achieve economically viable production of lipids from microalgae, cultivation optimization is an important factor to genetically control the lipid metabolism. Numerous factors such as irradiance, temperature, salinity and nutrient availability influence the microalgal fatty acid profiles [14-18]. Nutrient deprivation is one of the common stresses encountered by microorganisms in nature [19]. Nitrogen depletion is reported to induce the fatty acid accumulation in several microalgae [2, 20]. The fatty acid profile under different treatments in microalgae was mentioned but few reports are available on detailed connection with fatty acid synthesis genes [21]. Gene expression profiling could provide a basic understanding of the molecular responses to nitrogen deprived lipid accumulation in microalgae [22]. Expression of DGTT 1 genes significantly at higher levels under nitrogen deprivation by *Chlamydomonas reinhardtii* was reported by Msanne *et al*. [23]. Nitrogen starvation has affected the fatty acid composition of *Ettlia oleoabundans* along with increased gene expression for fatty acid and TAG synthesis upon starvation [24].

CONCLUSIONS

The absence of nitrogen source in the growth medium has induced the expression of fatty acid gene in *Chlorococcopsis minuta*. The up regulation of accD gene when compared to beta actin gene under nitrogen sufficient indicated the role of nitrogen stress in increasing the fatty acid production by microalgae. Because of the high potential of microalgae as a biodiesel feedstock, detailed characterization of genes crucial in fatty acid biosynthesis is of particular importance. The accD gene could be a potential candidate for better quality and higher production of fatty acids for microalgae based biofuel using metabolic engineering techniques.

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