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COMPARISON OF SEVEN HOUSEKEEPING GENES EXPRESSION IN MICROALGAE (*Chlorella vulgaris*) GROWN UNDER NITROGEN LIMITED CONDITION

JESSREEN TEGA[1], NOR 'AWATIF CHE SOH[1], SITI NURUL AMIRA NOOR AZMAN[1], CHA THYE SAN[1,2] and MALINNA JUSOH[1,2*]

[1]*Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[2]*Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[*]*E-mail: malinna@umt.edu.my*

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ABSTRACT

The study of the lipid synthesis pathway and the gene expression analysis of microalgae is crucial to understand more about the genes that play roles in lipid synthesis. The use of real-time polymerase chain reaction (PCR) in gene expression analysis is the best choice and it necessitates housekeeping genes (HKGs) as internal standard controls. It is essential to establish stable reference genes for the proper normalization. This study aimed at evaluating the stability of seven HKGs of microalgae, *Chlorella vulgaris* which were actin, alpha-tubulin, beta-tubulin, elongation factor, cyclophilin, 18S rRNA, and GAPDH that were grown under nitrogen-limited condition. *C. vulgaris* were grown in complete F/2 media with $NaNO_3$ concentration of 8.82 x 10^{-4} M for control and 2.2 x10^{-4} M (25%) of $NaNO_3$ for the nitrogen-limited condition. The cultures were grown for 28 days and RNA extraction was done at intervals of 7 days. Cycle threshold (Ct) values of control and treatment were generated from real-time PCR to evaluate the stability of HKGs. Results showed a significant stable expression of cyclophilin (28.04 ± 1.10 for control and 29.17 ± 1.06 for treatment) and GAPDH (37.23 ± 0.30 for control and 36.44 ± 0.53 for treatment). Meanwhile, other HKGs showed significant unstable expression. Thus, cyclophilin and GAPDH can be used as a reference gene of *C. vulgaris* for lipid production.

Key words: Housekeeping gene, nitrogen limitation, microalgae, *Chlorella*, *Chlorella vulgaris*, real-time PCR

INTRODUCTION

Microalgae have various applications namely in the food industry, bioplastics, biofuels, biobased chemicals, pharmaceuticals, cosmetics, and others (Koller *et al*., 2014). Currently, microalgae have received attention to become alternative fuel due to the depletion of fossil fuel. Microalgae have the potentials to supply sustainable fuel due to high biomass production rates and effective photosynthetic efficiency as compared to oleaginous plants such as soy, rapeseed, and African palm that have also been commercially used for biodiesel production (Robles-Heredia *et al*., 2015). Microalgae can produce more lipids under nitrogen-limited conditions by altering the metabolic pathways and lipids produced can be used for biodiesel (Miao & Wu, 2006). More recently, a combination of phytohormones and nitrogen-limited conditions was used to enhance biomass and lipid production in microalgae (Mandal *et al*., 2020). Hence, many studies had been done to investigate the pathways of lipid and gene expressions in microalgae and to identify the genes that are responsible for lipid metabolism pathway in microalgae (Ikaran *et al*., 2015; Li *et al*., 2016).

The best method to study gene expression is using real-time polymerase chain reaction (PCR) (Barber *et al*., 2005). Real-time PCR has several advantages over traditional methods such as it has high accuracy quantification and sensitivity to measure mRNA levels (Huggett *et al*., 2005). Cycle threshold (Ct) corresponds to several cycles for the PCR amplification to reach a fixed threshold and determine an increase in fluorescence intensity exponentially (Rosic *et al*., 2011).

* To whom correspondence should be addressed.

To quantify the target genes of interest in gene expression, the data need to be normalized to the reference genes or known as 'housekeeping genes'. The appropriate housekeeping genes (HKGs) are necessary so that accurate and valid results of target genes quantification can be achieved (Barber *et al*., 2005). HKGs are a group of genes that are continuously expressed and they are essential in maintaining basic cellular function (Zhu *et al*., 2008). There are several HKGs commonly used such as 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, actin, and β-tubulin (Thellin *et al*., 1999). GAPDH is involved in the glycolytic pathway, actin and β-tubulin are responsible for the cytoskeletal organization and cyclophilin are important in protein folding (Rosic *et al*., 2011).

There are several criteria required to be met by a gene to be regarded as a reliable HKG. The gene expression level should not be affected by experimental factors and expressed at a stable and constant level. Its expression between tissues and the physiological state of the organism should show minimal variability. It is desirable to pick such a reference gene that would show a similar Ct with the gene of interest. It is also important for HKG to be able to show the variability resulting from improper handling procedures and technical problems (Chervoneva *et al*., 2010).

There is inadequate validation of HKGs as reported by several groups of molecular biologists (Guenin *et al*., 2009; Kozera & Rapacz, 2013; Ambroise *et al*., 2019). In one example by Guenin *et al*. (2009), they found that only 32% among 188 real-time PCR analyses conducted appropriate validation of HKGs in their research. Most of them are usually acquired from previous publications. The researchers did not realize that there is no definition of a 'one-size-fits-all' gene for HKGs in normalizing the gene expression. Furthermore, gene stability that is obtained in particular experimental conditions is only appropriate for that experiment and cannot be applied to all other conditions. Therefore, in this study, the experiment focused on seven HKGs of microalgae, *Chlorella vulgaris* which were actin, alpha-tubulin, beta-tubulin, elongation factor, cyclophilin, 18S rRNA, and GAPDH that were grown in nitrogen-limited condition to evaluate the gene stability among them. Thus, the most stable HKG can be used in the study of gene expression of microalgae lipid production in the future.

MATERIALS AND METHODS

Microalgae mother cells culture condition

The inoculum was prepared by inoculating a single colony of microalgae, *C. Vulgaris* cells grown on F/2 agar plate into 500 mL of F/2 medium of three replicates. F/2 medium was made up of filtered seawater, autoclaved, and enriched with macronutrients ($NaNO_3$ and NaH_2PO_4. $2H_2O$) and micronutrients (trace elements and vitamins) (Guillard & Ryther, 1962). The concentration for $NaNO_3$ and NaH_2PO_4. $2H_2O$ that were used were 8.82 x 10^{-4} M and 3.62 x 10^{-5} M, respectively. Fifteen ppt of seawater salinity was used. The cultures were maintained at 24°C (±2°C) under continuous white light at a photon flux density of 80 µmol m^{-2} s^{-1} for two weeks. Next, the cultures were scaled up to 1.5 L to increase the cell volume and were aerated constantly with filter-sterilized air via 0.22 µm filter under the same conditions and incubated to reach stationary phase within 2 weeks.

Determination of cell number

Five hundred µL of microalgae culture was transferred into a 1.5 mL tube. Next, the culture was gently vortexed and pipetted a few times to get a uniform suspension of the cells. Haemocytometer (Neubauer) pre-affixed with a coverslip was used to count the cells by loading 10 µL of the cells suspension. The counting of the cells was done under a microscope with 400 x magnification.

Culture condition for nitrogen treatment and control

The initial cell density of each experiment was standardized at 9.3×10^7 cells in 1.5 L F/2 medium (15 ppt) and was prepared in a 2 L conical flask. A total of 30 conical flasks were prepared for both control and treatment conditions. As for the control condition, the medium was supplemented with complete F/2 nutrient with $NaNO_3$ concentration of 8.82 x 10^{-4} M. Meanwhile, for the treatment condition 2.2 x10^{-4} M of $NaNO_3$ concentration was used in F/2 media which was 25% of the control concentration. The cultures were incubated under the cool-white fluorescent lamp at a photon flux density of 80 µmol m^{-2} s^{-1}, at 24°C (±2°C) of temperature and were aerated with sterilized air. All cells were harvested for RNA extraction at Day After Treatment (DAT)-0, 7, 14, 21, and 28 from three replicates of control and treatment conditions.

Microalgae cell harvesting

All cells collected at each DAT were centrifuged at 13 000 rpm for 1 min at 4°C. The supernatant was removed and the pellets were washed twice with 500 µL diethyl dicarbonate (DEPC) water. Then, pellets were re-suspended in 100 µL of DEPC water and stored at -80°C until further use.

RNA isolation

RNA was isolated according to the GF-1 Total RNA Extraction kit (Vivantis) protocol. Approximately 100 mg of frozen pellets were ground to a fine powder using a pre-chilled mortar and pestle. Next, the ground material was placed into a cold, RNase-free 2 mL microcentrifuge tube. Four hundred µL of TR Buffer was added to the ground material and vortexed vigorously to mix them properly. The sample was centrifuged at maximum speed for 3 min. The lysate was then transferred into a homogenization column that was pre-assembled in a collection tube and centrifuged again at maximum speed for 2 min. The flow-through was saved and 350 µL of 80% ethanol was added to it. The mixture was pipetted a few times to mix them thoroughly. About 650 µL of the sample including any precipitate was transferred into an RNA Binding Column pre-assembled in a collection tube and was centrifuged at 10 000 x g for 1 min. The flow-through was discarded. Then, 500 µL of Wash Buffer was added into the collection tube and centrifuged at maximum speed for 1 min. Flow-through was discarded. Seventy µL of DNase I Digestion Mix was pipetted directly onto the membrane of RNA Binding Column and was incubated at room temperature for 15 min. Next, 500 µL of Inhibitor Removal Buffer was added onto the membrane before centrifuged at maximum speed for 1 min. The flow-through was discarded. Wash Buffer, 500 µL was added onto the membrane and was centrifuged at 10 000 x g for 1 min before discarding the flow-through. The step of washing with Wash Buffer has been repeated once again. After the flow-through was discarded, the column was centrifuged at 10 000 x g for 1 min to remove traces of buffer. Then, the column was placed into a new microcentrifuge tube before adding 50 µL of RNase-free water directly onto the membrane and incubated for 1 min. Lastly, the column was centrifuged at 10 000 x g for 1 min and RNA was stored at -80°C. A Biodrop spectrophotometer was used to quantify RNA concentration and purity.

cDNA synthesis

cDNA synthesis was carried out using the Thermo Scientific RevertAid First Strand cDNA synthesis procedure. RNA of about 200 ng was mixed in 100 µM Primer Oligo $(dT)_{18}$ (1 µL), 10 mM dNTP mix (2 µL), 5x RT Buffer (4 µL), 20 U/µL RiboLock RNase inhibitor (1 µL), 200 U/µL RevertAid M-MuLV RT (1 µL) and nuclease-free water to make up the total reaction mix to 20 µL. The preparation of priming premix was done on ice in an RNase-free reaction tube. The complete mixture of the reaction was incubated in the heating block according to the manufacturer's protocol; 42°C for 60 min to allow annealing of primers. Then, the mixture was incubated at 70°C for 5 min to terminate the reaction and later chilled on ice. The reaction was stored at -20°C for further use. cDNA produced was used as a template in the real-time PCR analysis.

Real-time PCR analysis

Real-time PCR was performed using QPCR Green Master Mix Lrox, 2x. Final real-time PCR reaction solution of 20 µL was carried out that comprised of 25 ng of cDNA template (2.5 µL), 0.8 µL of 10 µM of each primer (forward and reverse primer) (Table 1), and 10 µL of QPCR Green Master Mix Lrox. The real-time PCR parameters were set according to the manufacturer's protocol: 95°C for 2 min for initial activation, 95°C for 15 sec to unwind the DNA stands, 60°C for 30 sec to allow annealing of primer, and 72°C for 30 sec for the extension process. Forty cycles were set up to amplify the DNA.

Data quantification

The Ct values of control and treatment were compared to evaluate the stability of gene expression of HKGs. Data were further analyzed using a two-way analysis of variance (ANOVA) combined with Tukey HSD analysis, with p value set at 0.05, using IBM SPSS 20.0 (Statistical Product and Service Solutions).

RESULTS AND DISCUSSION

The use of real-time PCR is the best choice to study gene expression analysis. However, to obtain the reliable result of relative real-time PCR, it is essential to validate the stability of HKG as an internal standard control (Dean *et al*., 2002). Based on this study, cyclophilin and GAPDH showed the most stable HKG as compared to others (Figure 1). It means that the expression level of both cyclophilin and GAPDH of *C. vulgaris* showed minimal changes in control and nitrogen-limited condition and appropriate to be used as reference genes in gene expression study of the same experimental condition. These findings support the criteria that need to be met by a gene to be regarded as reliable HKG (Nicot *et al*., 2005; Chervoneva *et al*., 2010; Kozera & Rapacz, 2013). The transcription

Table 1. The primers sequence of HKGs used for real-time PCR

Gene	Orientation	Sequence (5'→3')
Actin	Forward	GAGAAGACCTACGACCTCCC
	Reverse	TCGCACTTCATGATGGTCTG
Alpha Tubulin	Forward	ACGGCAAGAAGTCAAAGCTC
	Reverse	CTCGTTGTCGAGCATGACTG
Beta Tubulin	Forward	AAGAACATGATGTGCGCC
	Reverse	GGGGATCCATTCCACAAA
Elongation Factor	Forward	CTCGCGGTCAAGTCGGTGCAG
	Reverse	CAGCGCACCGTGCTGTGCATG
Cyclophilin	Forward	CTTCCGCGCTCTGTGCACTG
	Reverse	GCCGTAGATGGACTTGCCGCC
18S rRNA	Forward	CCTGCGGCTTAATTTGACTCAACACG
	Reverse	TAGCAGGCTGAGCTCACGTTCG
GAPDH	Forward	TGGACGAGAAGTTTGGCATT
	Reverse	GAACGATGTTCAGGGCTGC

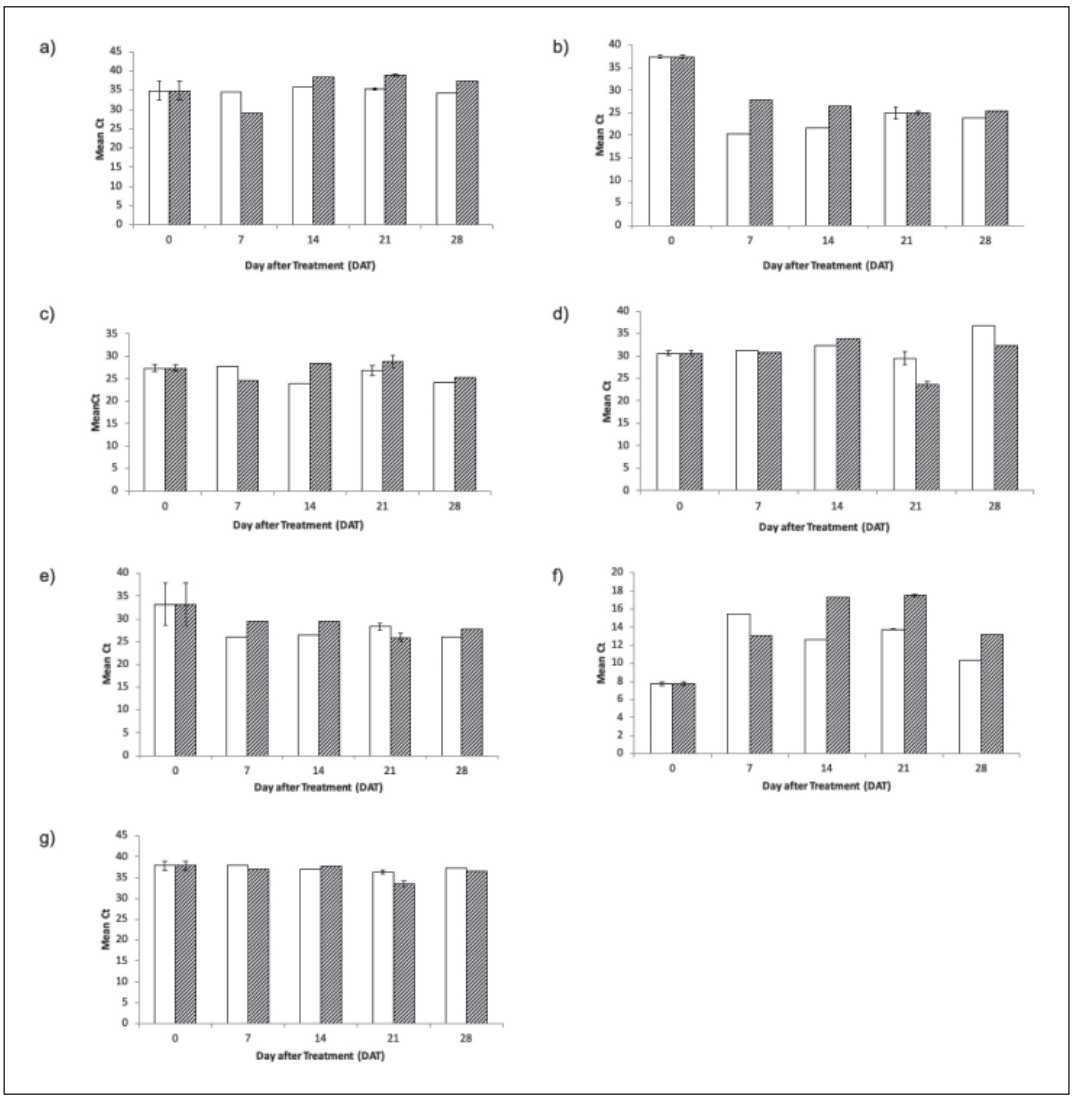

Fig. 1. Comparison of cycle threshold (Ct) values between control (white bars) and nitrogen-limited condition (shaded bars) 28 Days After Treatment (DAT) for actin (a), alpha-tubulin (b), beta-tubulin (c), elongation factor (d), cyclophilin (e), 18S rRNA (f) and GAPDH (g). Bars show the mean Ct values ± SE.

level of cyclophilin was lower than 35 cycles and GAPDH was higher than 35 cycles. According to previous studies, the Ct value of HKG below 35 cycles was recommended as a reference gene due to the high transcription level of target genes (de Kok *et al*., 2005; Rosic *et al*., 2011). However, Ct value above 35 still can be regarded as a potential reference gene under particular experimental conditions because the suitability of HKG will change depending on the stress factors (Nicot *et al*., 2005; Rosic *et al.,* 2011). A previous study had analyzed nine potential HKGs including GAPDH of diatoms, *Pseudo-nitzschia multistriata,* and *Pseudo-nitzschia arenysensis* that grown in nitrogen-limited condition. The result showed that GAPDH appeared stable for *P. arenysensis* but the unstable expression in *P. multistriata* (Adelfi *et al*., 2014). Thus, it is proven that the stability of HKG also relies on the types of the sample even though the stress factor of the experiment is similar.

GAPDH is the most popular HKG used in human gene expression studies (Tricarico *et al*., 2002; Barber *et al*., 2005). GAPDH plays an important role in the glycolytic cycle. It converts glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate (Sirover *et al*., 2014). However, GAPDH is rarely used in microalgae. The common HKG that has been used in microalgae gene expression studies is 18S rRNA (Jusoh *et al*., 2015; Li *et al*., 2015; Norashikin *et al*., 2018). From this study, 18S rRNA was highly expressed but it is unsuitable to be used as an internal standard control in relative real-time PCR. The expression of 18S rRNA of *C. vulgaris* was regulated by nitrogen-limited condition and the Ct value varies between 2 to 10 cycles (Figure 1). The previous studies which were following our results found that 18S rRNA was regulated by an experimental condition such as in the study of longan (Lin & Lai, 2010) and the study of 12 HKGs of microalgae, *Nannochloropsis* sp. in the diurnal cycle, low temperature, high and different light intensity conditions (Cao *et al*., 2012). The results showed that 18S rRNA was very stable in low-temperature conditions but unstable in other experimental conditions. Thus, 18S rRNA is not recommended to be used as a reference gene in relative real-time PCR.

Recent studies had suggested that at least two HKGs are needed to normalize gene expression in real-time PCR (Rosic *et al*., 2011; Cao *et al*., 2012; Guo & Ki, 2012; Ambroise *et al*., 2019) because it can increase resolution and accuracy of the result as well as the more valid result can be achieved (Thellin *et al*., 1999; Ambroise *et al*., 2019). However, a few factors need to be considered if multiple reference genes are to be used in an experiment such as time, cost, availability of RNA sample, and large dataset (Lin & Lai, 2010). In exceptional conditions, a single HKG is acceptable to be used if only it was tested in similar experimental conditions and properly validated previously (Thellin *et al*., 1999). In conclusion, two HKGs that are most suitable to be used as reference genes for the nitrogen-limited condition are cyclophilin and GAPDH.

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