Biocatalysis and Agricultural Biotechnology 17 (2019) 571–575

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Transcript analysis of hydrogenase A in an indigenous microalga, *Coelastrella* sp. M-60

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ABSTRACT

Biological hydrogen production by means of microalgae is one of the developing and potential clean fuels with future scope. In this study, the presence of hydrogenase A gene in the green indigenous microalgal isolate, *Coelastrella* sp. M-60 similar to the *hyd*A coding sequences of other green microalgae which are shown to have the potential to produce biohydrogen was taken into consideration. Differential gene expression study of hydrogenase A of *Coelastrella* sp. M-60 through real-time PCR in different culture conditions showed 33 folds upregulation of *hyd*A gene in partially anaerobic sulphur deprived conditions relative to the complete nutrient conditions.

1. Introduction

A rapid increase in population, energy demand and environmental concerns emphasize the necessity of renewable energy sources (Chisti, 2007; Gupta et al., 2016; Jaiswar et al., 2017). Microalgae serve as the potential alternative renewable feedstock for biofuels such as biodiesel (Chisti, 2007; Karpagam et al., 2015a), bioethanol and biohydrogen production (Mathimani and Pugazhendhi, 2018; Chi et al., 2019). Hydrogen has been found to be a promising alternative fuel to conventional fossil fuels, as it releases energy explosively without air pollutants in combustion (Debabrata, 2009). Out of chemical and biological synthesis of hydrogen, biological production is gaining importance due to its eco-friendliness compared to thermo-chemical process (Show et al., 2018; Dhanasekar and Jonesh, 2018). In general, photosynthesis is the fundamental driving force that supports all biofuel synthetic processes, converting solar energy into biomass, carbon storage products (e.g. carbohydrates and lipids), and/or H_2 in algae and plants (Beer et al., 2009). Biohydrogen production majorly depends on water splitting phenomenon by the microorganism by the effect of light, for which they derive electrons from the photosynthesis process (Manish & Banerjee, 2008). Hydrogen production from microalgae is being considered and developed as a potential clean stream of fuel for the future (Nguyen et al., 2008). Hydrogenases such as [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenase are the key enzymes in algae responsible for hydrogen production (Sharma and Arya, 2017). Biohydrogen is evolved by the special type of highly active [Fe]-hydrogenases that are encoded by inducible *hyd*A genes of algae during anaerobiosis (Happe et al., 2002). It was found that sulphur deprivation in some green microalgal species had produced a significant amount of H_2 due to the anaerobic/microaerobic conditions created through the strong reduction in PS II activity and CO_2 fixation process. As a response to anaerobiosis, the unicellular green alga *Chlamydomonas reinhardtii* and many other algae produce, molecular hydrogen (H_2) as one key metabolite using hydrogenases. Sulphur depletion inhibits the repair of PSII reaction center protein (D1) after photodamage, which consists of methionine residues (Melis et al., 2007; Nguyen et al., 2008). Therefore, when organisms are allowed to grow into anaerobic or hypoxic conditions, the normal metabolism might be altered which is correlated by altered gene expression, generally regulated on the transcription level as represented by large changes in transcript abundances in various organisms (Mustroph et al., 2010). Subsequently, this study reports the presence of an important gene candidate (*hyd*A) gene in the indigenous potent microalgae, *Coelastrella* sp. M-60 for the first its kind. Moreover, the differential expression pattern of *hydA* under different sulphur deprivation conditions along with complete nutrient condition was studied, which would provide clues to metabolic regulation of hydrogenase gene expression in microalgae.

2. Materials and methods

2.1. Culture conditions maintained for the induction of hydrogenase gene

Coelastrella sp. M-60, a freshwater indigenous microalgal isolate used in the study has been characterized and studied previously for

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https://doi.org/10.1016/j.bcab.2019.01.011
Received 20 November 2018; Received in revised form 20 December 2018; Accepted 4 January 2019
Available online 06 January 2019
1878-8181/

Table 1
Culture conditions maintained for the analysis of *hyd*A expression in *Coelastrella* sp. M-60.

S. No.	Culture conditions	Representation
1.	Control aerobic cultures in normal photoperiod in BG−11 medium (complete nutrient)	C
2.	Aerobic cultures in sulphur deprived BG−11 media	CD
3.	Aerobic cultures in dark in 0.2% glucose supplemented sulphur deprived media	CDG
4.	Partial anaerobic in sulphur deprived media	PA
5.	Partial anaerobic in 0.2% glucose supplemented sulphur deprived media	PAG
6.	Anaerobic conditions created in sulphur deprived media	SA
7.	Anaerobic in glucose supplemented sulphur deprived media	SAG

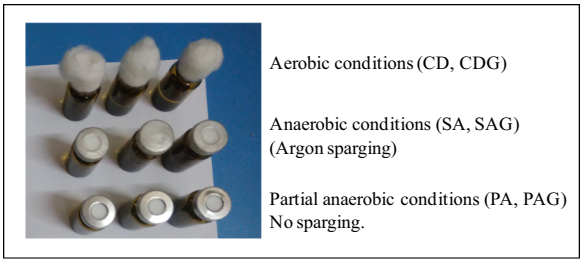

Fig. 1. Culture bottles maintained for the analysis of hydrogenase A expression in *Coelastrella* sp. M-60.

Table 2
Primer sets used for real-time PCR analysis.

S. No.	Gene	Primer Sequences	Product size (bp)
1.	*18 S rRNA*	FP- 5′CCTGCGGCTTAATTTGACTC 3′ RP- 5′ATCAACCTGACAAGGCAACC 3′	142
2.	*hydA*	FP- 5′AGGCGCAGTCATCAAGAACT 3′ RP- 5′ GTCGTGTTGAACCACTCACG 3′	126

lipid and carotenoid accumulation (Karpagam et al., 2015b, 2018). In this study, *Coelastrella* sp. M-60 was cultured till late logarithmic phase in BG-11 medium (represented as a complete nutrient) at 25 °C in alternate photoperiod (12 h light: 12 h dark) at 1500 lx light intensity, mentioned as C (control culture condition). Moreover, in order to induce hydrogenase gene expression, these cells of control culture condition, C were harvested, washed and incubated in S-deprived BG-11 media by keeping in a shaker at 100 rpm for 48 h in the above-mentioned light conditions. After incubation, the cells were harvested. About 1.9×10^8 cells mL^{-1} (equivalent to OD_{600}~ 5.5) were inoculated and maintained in amber colored bottles (13 mL capacity) containing 5.5 mL S- deprived BG-11 media. The culture conditions were named as CD, CDG, PA, PAG, SA, SAG; incubated in dark for 12–14 h and its representation is given in the following Table 1 and Fig. 1. For CD, CDG, aerobic condition was maintained by closing with cotton plugs. For SA and SAG, argon was sparged to create anaerobic condition whereas no argon was sparged for PA and PAG. Tightly sealed caps with crimpers were used to close culture bottles for SA, SAG, PA and PAG. This would prevent gas exchange in order to create anaerobic/ partially anaerobic conditions.

2.2. Amplification of hydA from Coelastrella sp. M-60

Total RNA was extracted from *Coelastrella sp.* M-60 grown in S deprived BG 11 medium and DNAase treated as per the protocol mentioned in Karpagam et al. (2018). cDNA synthesis was performed with 4 μg of DNAase treated RNA using random hexamers and oligodT primers provided in the revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA) using following conditions for PCR analysis: 5 min at 25 °C; 60 min at 42 °C and finally 5 min at 70 °C. Primers, HYD FP- CCACTGCCAATGTTCACAAGCTGC and HYD RP- AGCGGCCTCCA TCACGCCTCC specific to *hyd*A were designed based on conserved aminoacid sequences of Fe hydrogenases of microalgae and *Arabidopsis.* (Supplementary fig. 1).

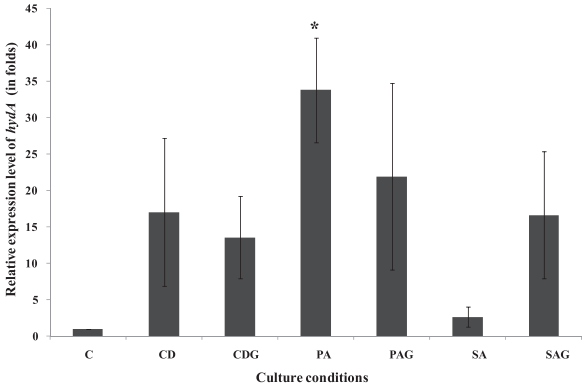

Fig. 2. Relative expression level of hydrogenase A (in folds) under different culture conditions (culture conditions described in Table 1 in materials and methods Section 2.1), calculated by real-time PCR experiments. Data represents the mean of triplicate experiments and error bar denoted the standard error (P * < 0.05).

2.3. Transcript analyses

Total RNA was extracted from the cells of above-mentioned culture conditions (in materials and methods Section 2.1), DNAase treated and cDNA was prepared as mentioned above. In order to analyze the gene expression pattern, the quantitative real-time PCR analysis was performed. Primers were designed from the hydrogenase A sequence of *Coelastrella* sp. M-60 (Genbank Acc. No. MH 899117) using the PRIMER 3 tool under default parameters (Table 2). Real-time PCR was performed using ABI PRISM® 7000 thermocycler (Applied Biosystems, USA) and SYBR Green Master mix (Roche, Germany) based on the manufacturer's protocol. 18S rRNA was used as the reference or housekeeping gene for normalization of *hyd*A gene expression. The relative gene expression level for each gene (in folds) was calculated by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Table 3
BLAST analysis of partial *hyd*A cDNA sequences of *Coelastrella* sp. M-60.

Query sequences	Length (bp)	Similarity with the nearest recorded *hyd*A sequences	Query Coverage	Maximum Identity
Coelastrella sp. M−60 (partial cDNA)	390	*Tetraspora* sp. CU2551	98%	89%
		Chlorella sp. DT	98%	85%
		Chlorella fusca	98%	84%

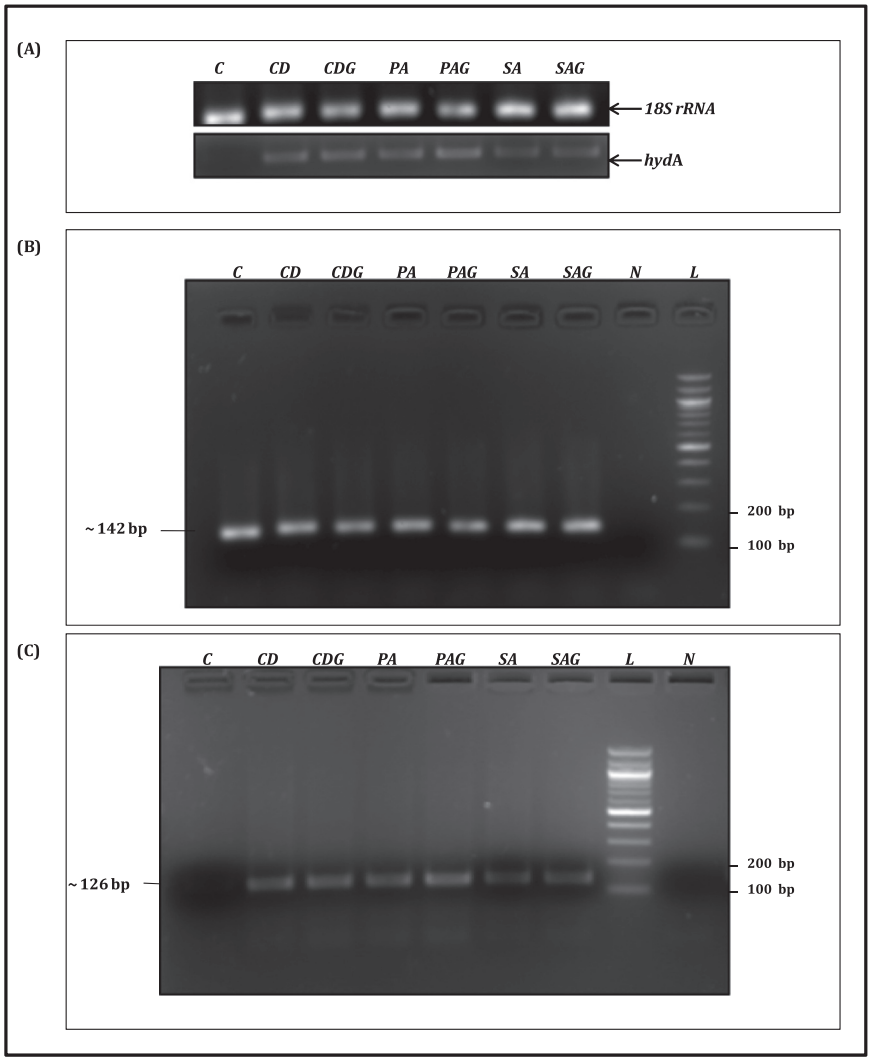

Fig. 3. (A) Semi- quantitative RT-PCR analysis of *hyd*A of *Coelastrella* sp. M-60: The expression of *hyd*A (after 28 cycles) was normalized to the internal standard (18S rRNA gene) through the intensities of respective bands using Image J tool. (B) Amplification of 18S rRNA gene of amplicon ~ 142 bp. (C) Amplification of *hyd*A gene of amplicon ~ 126 bp.

3. Results and discussion

3.1. Sequence confirmation of hydA in Coelastrella sp. M-60

In algal species, H^+ via chloroplast hydrogenase (*Hyd*A) encoded from nuclear genome can accept electrons directly from ferredoxin and generates H_2 (Chien et al., 2012). In this study, hydrogenase A (*hyd* A) gene (partial) was amplified from the total cDNA of indigenous microalgal isolate, *Coelastrella* sp. M-60 from the primers designed as mentioned above in Section 2.2 and sequenced (supplementary fig. 2). The sequencing result on BLAST analysis confirms the sequences as partial *hyd*A cDNA sequences of *Coelastrella* sp. M-60 which was then submitted to Genbank [Acc. No. MH 899117]. The partial *hyd*A cDNA sequences of *Coelastrella* sp. M-60 showed the highest similarity to the hydrogenases mRNA sequences of other green microalgae species *Tetraspora* sp. CU2551 followed by *Chlorella* sp. DT and *Chlorella fusca* (Table 3).

3.2. Expression of hydA gene in Coelastrella sp. M-60 under different culture conditions

Expression of *hyd*A gene in *Coelastrella* sp. M-60 under normal conditions represented as 'C' was compared with different dark incubation conditions in partial anaerobic and anaerobic conditions created in S deprived and glucose supplemented sulphur deprived BG 11 medium were analysed through real-time PCR. Culture conditions are depicted in the Fig. 1 and Table 1. The real-time PCR results showed considerable upregulation in S- deprived medium especially in partial anaerobic conditions (PA) (33.8 ± 7 folds) and partial anaerobic with glucose supplementation (PAG) (21.9 ± 12.8 folds), when compared with the expression of *hyd*A under aerobic complete nutrient conditions (C) (Fig. 2). It is further observed that CD, CDG, SA, SAG also had induced *hyd*A gene expression noticeably with 17 ± 10, 13.6 ± 5.7, 2.7 ± 1.4 and 16.6 ± 8.7 folds respectively. Dissociation curves, cycle threshold values for 18 S rRNA and *hydA* obtained from real-time PCR experiments are given in Supplementary fig. 3. Further in this study, semi- quantitative PCR analysis substantiates the considerable upregulation in sulphur deprived medium adopted with the above mentioned media conditions than the normal complete nutrient conditions represented as C (Fig. 3).

On the other hand, higher *hyd*A upregulation in partial anaerobic condition (PAG, PA) (no argon sparged) than the anaerobic (argon sparged) (SA, SAG) was observed in this study (Fig. 2). This is likely due to the combined effect of the stress on cell growth imposed simultaneously by S deprived culture conditions and sparging of gas in the small culture vial. In this way, Rühle et al. (2008) state that drastic

metabolic changes during nutrient stress such as prolonged S deprivation results in algal cell growth inhibition. Moreover, Yang and Wang (1992) and Camacho et al. (2001) emphasized the effect of hydrodynamic stress in cell inactivation in the presence of sparging and agitation. If the mechanism of S-deprivation based hydrogen production in microalgae is considered, during S deprivation oxygenic photosynthesis decreases, while respiration induces anaerobic condition appropriate for hydrogen production (Khetkorn et al., 2017). Gabrielyan et al. (2017) reported that different carbon sources such as glucose, acetate, and fructose had a positive effect in hydrogen production in *Parachlorella kessleri*, RA-002. Moreover, there would be a transcriptional level regulation on the expression of hydrogenases during the carbohydrate catabolism in the cells (Posewitz et al., 2009). Subsequently in this study, 0.2% glucose supplementation was provided in S deprived media as the carbon source that resulted in the induction of hydrogenase gene expression.

It was also reported that HYDA1 transcript increases in algae, when it was transferred to anaerobic conditions at dark (Happe and Kaminski, 2002) or in illuminated, S deprived algae (Hemschemeier et al., 2008). Chien et al. (2012) reported that *hyd*A transcription activity was observed only under anaerobic or sulphur-deprived conditions in *Chlorella* sp. DT. Whereas, Winkler et al. (2002) characterized the activity of [Fe]-hydrogenase (*hyd*A) of *Chlorella fusca* under anaerobic conditions which was sparged with argon in the dark. In contrary, in the case of *Tetraspora* sp. CU 255, Maneeruttanarungroj et al. (2012) demonstrated the different response to sulphur deprivation between *C. reinhardtii* and *Tetraspora* sp. CU 255, wherein the production of H_2 and PSII activity was decreased in cells grown under sulphur-deprived condition and these two activities were restored when the cells transferred to the medium supplemented with sulphur.

Induction of anaerobic or hypoxic conditions in *C. reinhardtii* cultures grown in sulphur (S)-free medium produced a significant amount of hydrogen (Melis et al., 2000; Hemschemeier et al., 2009). This can be attributed to the fact that PSII and carbon dioxide fixation activities decrease strongly, resulting in anaerobic (or microaerobic) conditions and a rigorous reduction of electron sinks of both respiratory and photosynthetic pathways (Hemschemeier et al., 2008; Melis, 2007; Hemschemeier and Happe, 2011). Under these conditions, HYDA1 acts as an alternative electron acceptor of photosynthetic electron flow. However, small but significant amounts of H_2 are also produced by anaerobically adapted *C. reinhardtii* cultures in the dark (Gfeller and Gibbs, 1984; Philipps et al., 2011).

4. Conclusion

Thus this study reports the presence of *hyd*A gene and its differential expression under different culture conditions in an indigenous microalgal isolate *Coelastrella* sp. M-60 for the first time. The future scope of this work is the standardized system for sustained biohydrogen production using *Coelastrella* sp. M-60.

Acknowledgement

Authors thankfully acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi, India for the financial support to R. Karpagam (File no. 09/201/0418/2016-EMR-I dated 31.3.2017) as Research Associate and to Dr. P. Varalakshmi as research supervisor. We also acknowledge DST-PURSE, UGC- SAP, UGC-UPE and Central Instrumentation facility of Madurai Kamaraj University, Madurai for the instrumentation facilities.

Author contributions

All authors are contributed to the manuscript.

Conflict of interest

Authors declare no conflict of interest

Competing financial interests

The authors declare no competing financial interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2019.01.011.

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