Transcription of the Hydrogenase Gene during H₂ Production in *Scenedesmus Obliquus* and *Chlorella Vulgaris*

Yahaira de Jesus Tamayo Ordóñez

Universidad Autónoma de Coahuila: Universidad Autonoma de Coahuila

Benjamin Abraham Ayil Gutiérrez

Instituto Politécnico Nacional: Instituto Politecnico Nacional

Alejandro Ruiz Marin

Universidad Autónoma del Carmen: Universidad Autonoma del Carmen

Francisco Alberto Tamayo Ordóñez

Universidad Autónoma del Carmen: Universidad Autonoma del Carmen

Ileana Maria Mayela Moreno Davila

Universidad Autónoma de Coahuila: Universidad Autonoma de Coahuila

Leopoldo Ríos González

Universidad Autónoma de Coahuila: Universidad Autonoma de Coahuila

Jose Antonio Rodriguez de la Garza

Universidad Autónoma de Coahuila: Universidad Autonoma de Coahuila

Juan Carlos Robles Heredia

Universidad Autónoma del Carmen: Universidad Autonoma del Carmen

Maria Concepcion Tamayo Ordoñez (✉ mtamayo@uadec.edu.mx)

Universidad Autonoma de Coahuila <https://orcid.org/0000-0003-0201-0184>

Original article

Keywords: microalgae, hydrogenase gene, molecular hydrogen, mutation

Posted Date: March 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-342043/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

There is ongoing research related to the production of molecular hydrogen today and algae have proven to be good biological models for producing several compounds of interest. We analyzed how genetic variations in hydrogenase genes (*hyd*) can affect the production of molecular hydrogen in the algae *Chlorella vulgaris* and *Scenedesmus obliquus*. Through isolation and genetic characterization of *hyd* genes in *S. obliquus* and *C. vulgaris*, we made in-silico 3D modeling of the hydrogenase proteins and compared these in 11 algal genera. The 3D structure of hydrogenases indicated its structural conservation in 10 genera of algae, and the results of our grouping according to the aa characteristics of the proteins showed the formation of two groups, which were unrelated to the algae's phylogenetic classification. By growing *C. vulgaris* and *S. obliquus* in anaerobic conditions (in darkness) during 24 h and after exposing the cultures to light, we observed H₂ production values of 9.0 ± 0.40 mL H₂/L and 16 ± 0.50 mL H₂/L, respectively. The highest global relative expression of *hyd* genes was reached during the first 30 min of exposure to light. The behavior of the expression of the *hyd* genes in these species of algae proved to be species specific and involved in the production of H₂. Future identification of isoforms of *hyd* genes in algae would allow a better understanding of the regulation of the hydrogenase enzyme.

Introduction

The pressure exerted on the environment by the demand for natural resources has promoted proposals for improving and developing new production and biotechnology models compatible with the development of alternative energies (Dincer and Acar 2015; Nikolaidis and Paullikkas 2017). Molecular hydrogen (H₂) is a fuel alternative due to its high energy content and its relatively clean combustion (Show et al. 2018; Srivastava et al. 2020). Currently, the global annual hydrogen production is approximately 0.1 GT, which is widely applied for refining and metal treatment, and a small fraction of it is used as automobile fuel (Nikolaidis and Paullikkas 2017; Sharma and Ghoshal 2015). H₂ is also used as a source of energy, and the generation of electricity by means of fuel cells is already in the expansion phase in the US and the UK, making its use as an energy source to become a reality (Staffell et al. 2019; Nagarajan et al. 2017).

The use of algae for H₂ production has been extensively studied (Fakhimi et al. 2020; Jimenez-Llanos et al. 2020; Lam et al. 2019; Nagarajan et al. 2017). Algae are organisms capable of using sunlight for water oxidation through oxygenic photosynthesis. In algae, the light energy absorbed during photosynthesis facilitates the oxidation of water molecules, the release of electrons and protons, and the endergonic transport of these electrons to ferredoxin. Then, the enzyme hydrogenase reduces the protons to hydrogen, oxidizing ferredoxin, which passes from its reduced state to the oxidized state (Rochaix 2020). The ability of algae to generate molecular hydrogen through oxygenic photosynthesis has led related research to run in two directions: the optimization of abiotic factors (pH, temperature, and light quality, among other parameters) during the growth of the algae (Díaz-Rey et al. 2015; Laurinavichene et al. 2006;

Rashid et al. 2013), and genetic engineering of enzymes involved in the oxidation of water during oxygenic photosynthesis (Khetkorn et al. 2017; Limongi et al. 2021; Santos-Merino et al. 2021).

Green algae have demonstrated their ability to decompose water into H_2 and O_2 aided by light energy. Two photosynthetic systems are involved in this process: photosystem I (PSI) and photosystem II (PSII). In PSII, photons derived from light energy split water into O_2 and electrons. The electrons are then activated in PSI, which will reduce ferredoxin (Fd). By the activity of hydrogenases, Fd (red) can be reoxidized, forming H_2 [FeFe]-hydrogenase mediates this reaction in algae (Milrad et al. 2018).

Photobiological production of H_2 seems to be an economically viable alternative; however, at present it has not yet been fully understood how the activity of enzymes involved in the production of molecular hydrogen in algae is regulated. Recently, attention has been given to hydrogenase enzymes. In green microalgae, [FeFe]-hydrogenases are enzymes localized in the chloroplast that release electrons from H_2 and oxidize molecular hydrogen into two protons and two electrons (Carrieri et al. 2011), whose activity has been reported to be induced through anaerobic adaptation in culture supplemented with argon or nitrogen and incubated in the dark (Florin et al. 2001; Happe and Kaminski 2002; Kim and Kim 2011). However, the induction of hydrogenases has been shown to be transient and regulated by O_2 and CO_2 , making it necessary to regulate the level of photosynthesis and cellular respiration of these algal systems (Carrieri et al. 2011; Kim and Kim 2011).

The literature contains more research reports about algal culture optimization than about genetic manipulation of genes involved in H_2 production in algae. Within this context, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Desmodesmus*, and *Chlorella* have been shown to have good H_2 production in sulphide-free media (Timmins et al. 2009). Furthermore, in *C. reinhardtii*, the high production of H_2 has been verified to be induced by sudden change from dark to light, so that the cells that had become anaerobic in the dark begin to express the hydrogenase gene in the presence of light; however, the effect only lasts a few minutes (Mus et al. 2005). In this system, the hydrogenase accepts electrons produced by photosystem II (PSII) until the activation of the Calvin cycle, and then, the hydrogenase is inhibited by the increasing concentration of O_2 in the medium. Apparently, the regulation of the activity of hydrogenase enzymes is related to three main pathways involving the regulation of photosystems, the respiratory chain, and the Calvin cycle.

Lakaniemi et al. (2011) reported H_2 production values of 7.9 and 10.8 ml H_2 /g volatile solids (VS) in *C. vulgaris* in dark fermentation conditions, and Sun et al. (2011) obtained maximum H_2 production values of 7.13 ml/g VS/144 h at an inoculum-substrate ratio of 0.3 in *Chlorella* sp. Tinpranee et al. (2016) reported *Chlorella* sp. LSD-W2 produced 66.47 ± 5.44 and 67.64 ± 1.77 nmol H_2 /mg dry weight at four days of culture in anaerobic conditions, variable light intensity using $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white-light radiation, and a temperature of 30°C . Azwar et al. (2009) reported H_2 production values of 1.35 ml $L_{\text{cult}}^{-1} \text{h}^{-1}$ in *C. sorokiniana* strain Ce cultured at a light intensity of $120 \mu\text{E/m}^2/\text{s}$.

In the case of *Scenedesmus* sp., Batista et al. (2014) observed H₂ production values of 90.3 mL/g ST in *Scenedesmus* sp. in cocultures with *Clostridium butyricum* during dark fermentation conditions, and Yang et al. (2011) obtained a H₂ production value of 49.5 cmL/g SV using *Scenedesmus obliquus* in mixed culture with untreated sludge.

Apparently, the response of algae to dark fermentation conditions, different light intensities and sudden change from dark to light, originates different responses regarding H₂ production (Xia et al. 2015). These different responses in algae could be influenced by the regulation of the hydrogenase enzyme, essential in photosystem II and regulated by the presence of light.

The activity of the enzyme hydrogenase has been detected in algae species – such as *Scenedesmus obliquus*, *Chlorococcum littorale*, *Platymonas subcordiformis*, *Chlorella fuscas*, *Chlamydomonas augustae*, and *Chlorella vulgaris* (Florin et al. 2001; Eroglu and Melis 2011; Srirangan et al. 2011)– and, despite the achievements made in the production of hydrogen in algae, it is estimated that the production rates achieved are around 15% of the theoretical biological maximum, thus suggesting that substantial improvements are required in the process (Melis 2002).

Genetic engineering has proven to be an excellent tool to increase H₂ production in algae. For example, through the heterologous expression of the hydrogenase operon (*hydA*, *hydB*, *hydE*, *hydF*, and *hydG*) of *Shewanella oneidensis* MR-1 into *Anabaena* sp. PCC 7120, a production of 3.4 nmol H₂ µg chl $\alpha^{-1}h^{-1}$ has been achieved (Khetkorn et al. 2013). Also, the homologous overexpression of the HydA gene in *Chlorella* has been shown to increase H₂ production 7-fold, compared to the unmutated strain (Dubini and Ghirardi, 2015), and the expression of a ferredoxin hydrogenase fusion enzyme (Fd-Hyd) in *Chlamydomonas reinhardtii* was shown to increase H₂ production 4.5 times more compared to the mutant strain without hydrogenase (Chien et al. 2012; Weiner et al. 2018). Knowing that hydrogenase genes in algae are apparently regulated during H₂ production could help to develop better genetic transformation methods aimed at optimizing H₂ production.

Our study was preceded by the report of Ruiz-Marin et al. (2020), who studied the response in H₂ production of *C. vulgaris* and *S. obliquus* under different intensities of white, violet, and blue light, showing H₂ production yields of up to 60.4 (mL/L) in *C. vulgaris* and of 128 ml/L in *S. obliquus* when cultured in the presence of violet light. Ruiz-Marin et al. (2020) highlighted that *C. vulgaris* showed a prolonged lag phase before hydrogen production of 70 h, 35 h, and 10 h under white, purple and blue light, respectively, and suggested that this time was required to change the metabolism from autotrophic to heterotrophic, enable the use the available carbon sources in wastewater, and trigger the subsequent expression of *hyd* for H₂ production. However, *S. obliquus* only presented a lag phase in cultures under white light, which the authors suggested was due to the high capacity of this species to activate *hyd* for H₂ production during the first hours of anaerobic conditions in darkness. Therefore, light intensity and *hyd* regulation could be species-specific according to each microalga's adaptive characteristics to the cultivation conditions; for that reason, in this research we characterized genetically the enzyme

hydrogenase in the algae *Chlorella vulgaris* and *Scenedesmus obliquus*, and evaluated the expression of these *hyd* genes in cultures optimized for H₂ production. The heterologous or homologous expression of *hyd* genes in algae could help to improve the yields of H₂ production in these organisms.

Materials And Methods

Obtention of algal biomass

Ruiz-Marin et al. (2010) isolated the microalgae used in this study, *S. obliquus* from hypereutrophic soil, and *C. vulgaris*, from agricultural soil. Currently these microalgae belong to the Microalgae Culture Collection of the Center for Scientific Research and Higher Education of Ensenada, Baja California (CICESE).

The microalgae were cultured in a sterile artificial wastewater medium (7 mg L⁻¹ of NaCl, 4 mg L⁻¹ of CaCl₂, 2 mg L⁻¹ of MgSO₄·7H₂O, 15 mg L⁻¹ of KH₂PO₄ and 115 mg L⁻¹ of NH₄Cl) (Ruiz-Marin et al. 2010). Trace metals and vitamins were added according to the f/2 medium (Guillard and Ryther 1962). The algal cultures were shaken (100 rpm) at 25°C, at an irradiance of 140 μE m⁻² s⁻¹. To obtain a cell density of 10×10⁷ cells/mL, the cultures were supplemented with 115 mg L⁻¹ of NH₄Cl and transferred to fresh artificial wastewater every 7 days, maintaining the cultures in agitation in an orbital shaker (100 rpm).

Isolation, identification, and phylogenetic analysis of 18SrDNA and *hyd* genes in algae

DNA was extracted from culture in suspension and through the DNAzol protocol (Chomczynski et al. 1997) and hydrogenase gene (*hyd*) amplification was conducted using the primers described for *Chlorella fusca* (Winker et al. 2002) and *Chlamydomonas reinhardtii* (Kamp et al. 2008, Qian et al. 2008, Forestier et al. 2003). The identification of the strains of *C. vulgaris* and *S. obliquus* was carried out by sequencing a partial region of 18S rDNA (Forestier et al. 2003).

PCR reactions were carried out in a volume of 50 μL containing 25 ng of genomic DNA, 130 μM dNTPs, 15 μM of each primer, 2.5 units of *Taq* DNA polymerase, and 1X PCR reaction buffer (Life Technologies, Rockville, MD, U.S.A.) with 1.5 mM MgCl₂. PCR conditions included one cycle of 3 min at 94 °C for initial denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at alignment temperature (tm) according to each set of primers used (Table S1), 1 min at 72 °C, and finally, 7 min at 72 °C. The PCR products were separated by electrophoresis in 1.2% agarose gels. Purification, cloning, and sequencing of PCR products was carried out according to Tamayo-Ordoñez et al. (2016).

The nucleotide sequences were aligned (BLASTX) and compared with those in the GenBank database. DNAMAN version 4.0 was used to translate these sequences and to identify the open reading frame. Predicted amino acid (aa) sequences relative to *hyd* nucleotide sequences were used in combination with

related sequences retrieved from GenBank to build a phylogenetic aligning. Conserved aligned regions (>90%) were selected in all sequences and phylogenetic analysis was performed in software MEGA version 6.0 (Kumar et al. 2016). Domains of interest for both proteins were identified using the conserved domain database (CDD) (Derbyshire et al. 2015).

Identification of haplotype and in silico modeling of the tertiary structure (3D) of hydrogenase enzymes in algae

Minimum haplotypes of Hyd enzymes were constructed using the median-joining method implemented in Network v.4.6 software (Bandelt et al. 1999), assuming an epsilon of 0 and a transversion/transition ratio of 1:2. Tertiary structure analysis of Hyd was performed with SWISS-MODEL (Biasini et al. 2014), Values of sequence identity (%), Global Model Quality Estimation and Qualitative Model Energy Analysis, were considered. As references, the sequence corresponding to [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* was included (Swanson et al. 2015).

Determination of H₂ in *Chlorella vulgaris* and *Scenedesmus obliquus*

Suspension cultures of *Chlorella vulgaris* and *Scenedesmus obliquus* with a cell density of 10×10^7 cells / mL were used for the preparation of immobilized algal beads according to Ruiz-Marin et al. (2020). Algal beads were formed by dropping a mixture of cells and 4% sodium alginate into a 2% calcium chloride solution using a 50 mL burette. With this method, Ruiz-Marin et al. (2010) reported that 2.5 mm in diameter algal beads were formed having an initial cell number of 3.2×10^5 cells/bead for every 100 mL of the algae-alginate mixture.

The cultures of immobilized *C. vulgaris* and *S. obliquus* microalgae were grown under white light for 4 days before transferring the microalgae-alginate-beads to reactors under anaerobic conditions in sulfate free medium (7 mg L⁻¹ of NaCl, 4 mg L⁻¹ of CaCl₂, 2 mg L⁻¹ of MgCl₂, 7 mg L⁻¹ of NaCl, 4 mg L⁻¹ of CaCl₂, 15 mg L⁻¹ of KH₂PO₄ and 115 mg L⁻¹ of NH₄Cl) supplemented with 10 g L⁻¹ glucose at pH 8 and a light intensity of 140 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Each reactor was placed inside the chamber, N₂ gas was purged into the medium for 10 min to remove dissolved oxygen and were kept in the dark for 24 h to achieve anaerobic conditions. Subsequently, the cultures were exposed to light (140 $\mu\text{E m}^{-2} \text{s}^{-1}$) and H₂ was measured at 4 h intervals for one day.

Hydrogen was measured using high-performance liquid chromatography (HPLC) according to Ruiz-Marin et al. (2020). The gathered data were subjected to statistical analysis by Tukey tests evaluated at P > 0.05. using the OriginPro 2020b software.

Determination of relative expression of *hyd* genes by qPCR

Because the expression of *hyd* genes and the enzyme activity are transient (Milrad et al. 2018; Khan and Fu 2020), induction of hydrogenase genes in cultures with 7 days of biomass growth was achieved by 24 h of dark incubation followed by immediate white light ($140 \mu\text{E m}^{-2} \text{s}^{-1}$) illumination of the reactors. The sampling of RNA extraction was made in two experiments, in the first one, samples were collected after 1, 5, 9, 12, 16, 20, and 24 hours of light exposure, and in the second, after 30, 60, 90, 120, 150, 180, 210, 240, 230, and 300 min of light exposure.

RNA isolation and cDNA synthesis were conducted according to Tamayo-Ordóñez et al. (2015). The *hyd* primers used for relative expression analysis were the same as described above (Table S1) and the 18S rDNA genes were used as reference genes according to Dong et al. (2012).

Amplifications of the *hyd* genes were carried out as described above in the same PCR amplification conditions used for the phylogenetic analysis. The melt curve analysis and negative controls for the reference and target genes were always included in the experiments in order to eliminate DNA contamination. The relative expression of each gene was determined by the $\Delta\Delta\text{Cq}$ method between the target (*hyd*) and reference (18S rDNA) genes by the equation: Relative expression = $(E_{\text{ref}})^{\text{Ct}_{\text{ref}}}/(E_{\text{target}})^{\text{Ct}_{\text{target}}}$ (Pfaffl 2001).

Results

The algal strains of *C. vulgaris* and *S. obliquus* were identified by our results of sequencing of a partial region of 18S rDNA. The amplified 135 bp of 18S rDNA partial regions from *C. vulgaris* and *S. obliquus* DNA had 100% identity with *Scenedesmus* and *Chlorella* accessions in the NCBI's GenBank (Fig. S1).

We also amplified a partial 700 bp (233 aa) region corresponding to the C-terminal domain of the iron hydrogenase large subunit. The identification of the *hydA* gene of *C. vulgaris* and *S. obliquus* provided us information about the genetic differences between phylogenetically distant groups of the algal hydrogenases, about non-synonymous changes in algal hydrogenases that could affect the protein's functionality, and about the possibility of differential values in the production of H_2 associated with the regulation in the transcription of the *hydA* gene in *C. vulgaris* and *S. obliquus*.

The hydrogenases of *Scenedesmus obliquus* and *Chlorella vulgaris* and their intra and interspecific evolutionary relationships with other algae

In order to establish genetic differences in the hydrogenase enzyme among algae, and to know if these non-synonymous changes affecting the 3D structure of the protein were related to the evolution of each

species in the genus, we made a bioinformatic analysis including dendrogram construction and 3D structure modeling of the enzyme in 24 accessions, including 11 algal genera.

The results from our phylogenetic analysis indicated the formation of a main group including the genera *Chlorella*, *Tetraspora*, *Scenedesmus*, *Tetradismus*, *Monoraphidium*, *Raphidocells*, *Chlamydomonas*, *Volvox*, and *Coccomyxa*. A second subgroup included the genus *Tetraselmis*. Some accessions of *Chlorella*, *Raphidocelis*, and *Nannochloropsis* formed a third group (Fig. 1), indicating that perhaps there are variants of hydrogenases in *Chlorella* and *Raphidocelis*.

The dendrograms resulting from grouping algal accessions according to the characteristics of their hydrogenases did not in all cases correspond to the organisms' phylogenetic classification. On one side, the genera *Chlorella* and *Coccomyxa* belong to the class Trebouxiophyceae but their accessions formed two subgroups within the main branch, similarly to the grouping pattern followed by accessions of *Volvox* and *Tetraspora*, both in the order Chlamydomonadales. On the other side, accessions of the genera *Scenedesmus* and *Tetradismus* –belonging to the family Scenedesmaceae– grouped together reflecting a close relationship, and this same grouping pattern occurred between accessions in the genera *Monoraphidium* and *Raphidocelis* both in the family Selenastraceae.

Our results from the 3D modeling of the HYD enzymes from *C. vulgaris* and *S. obliquus* showed found a 60% and 55% sequence identity with the [FeFe]-hydrogenase described for *Chlamydomonas reinhardtii*, respectively (Swanson et al. 2015) (Table S2). Almost all 3D models demonstrated similar structures, except that for *Coccomyxa subellipsoidea* XP_005643907.1 (Fig. 2, Fig. S2, Fig. S3). In almost all the 3D [FeFe]-hydrogenase structures modeled for the algal genera included in this study, we identified the aa residues responsible for the binding of the Iron/sulfur cluster (CPCACGCG; Fig. 2, Fig. S2, Fig. S3), but this sequence of residues was absent in the accessions *Coccomyxa subellipsoidea* XP_005643907.1 and *Tetradismus obliquus* AA65921.1. Interestingly, our results also showed that accession *Tetradismus obliquus* CAC34419.1 presented the binding aa sequence of the Iron/sulfur cluster, supporting the possible presence in algae of hydrogenase isoforms.

Isoforms of ferredoxins Fdx2–Fdx6 have been identified in *Chlamydomonas reinhardtii* that express differently depending on environmental conditions of oxygen, copper, iron, and ammonium concentrations (Meuser et al. 2011; Winkler et al. 2010) Furthermore, different isoforms of the genes coding for [FeFe] hydrogenases have been reported for *C. variabilis* (HydA1 and HydA2) and *C. reinhardtii* (HydE, HydF, and HydG). Also, studies of Bayesian inferred phylogeny reveal that the algal HydA are monophyletic and are nested between HydA genes of bacteria, fungi, and heterotrophic flagellates, which suggests that HydA emerged once early in chlorophyte evolution (Meuser et al. 2011) and also implies the possibility of finding genes coding for other isoforms of Hyd.

With the aim of knowing the variants of *hyd* genes, we built a network of haplotypes. For the construction of the minimal network of haplotypes of [FeFe]-hydrogenase we evaluated two structural domains in different microalgae species: the small subunit, and the terminal carbonyl domain (-C). The results of our evolutionary relationship analysis of both domains of the [FeFe]-hydrogenase in accessions of the genera

Chlamydomonas, *Nannochloropsis*, *Chlorella*, *Scenedesmus*, *Tetraselmis*, *Raphidocelis*, *Monoraphidium*, *Tetraspora*, and *Volvox* indicated the presence of 17 genetic variants supported by 235 (small subunit domain) and 232 (C-terminal) total mutations. The small subunit variants of the [FeFe]-hydrogenase showed 192 discrete mutations among the analyzed genera and 43 divergent mutations in *Chlorella* ADK77883.1 and *Tetraspora* AMY63159.1, designated as V7CHLO and V14CHLO. The behavior of the C-terminal domain was similar, with 190 discrete and 42 divergent mutations. Accessions *Chlorella fusca* CAC83290.1, *Tetradismus obliquus* CAC34419.1, *Monoraphidium neglectum* XP_013906846.1, and *Volvox carteri* f. nagariensis XP_0029484897.1 presented the fixed variant (Fig. 3a).

Furthermore, our mutational analysis of [FeFe]-hydrogenase indicated conservation of 120 aa residues among accessions from species of *Chlorella* (CAC83290.1, ADK77883.1, PRW60372.1, AEA34989.1) and *Scenedesmus* (AXU2407.1) –including the V3CHLO and V4SCEN sequences we identified in this study– and distinguished an interspecific relationship between the V15CHL (small subunit domain and C-terminal) and V9CHLO (small subunit domain) variants of *Chlorella* (Fig. 3a and b). The variants showing a close mutational relationship were V8TETRA (*Chlorella sorokiniana* PRW60372.1) and V13TET (*Tetradismus obliquus* AAG59621.1).

On the contrary, we observed a marked divergence among the V1CHLA (*Chlamydomonas reinhardtii* XP_00693376.1), V2NACH (*Nannochloropsis gaditana* XP_005854541.1), V5TETR (*Tetraselmis* sp. AHH85809.1), V11RAP (*Raphidocelis subcapitata* GBT94161.1), V12MON (*Monoraphidium neglectum* XP_013906846.1), V14TET (*Tetraspora* sp. AMY63159.1), V16NAN (*Nannochloropsis salina* TFJ80951.1), and V17VOL (*Volvox carteri* XP_002948487.1) variants (Peters et al. 2015; Sawyer and Winkler 2017)

Overall, this analysis demonstrates interspecific diversity of the [FeFe]-hydrogenase C-terminal and small subunit domains among microalgae taxa (Fig. 3). Interestingly, the evolutionary path of [FeFe]-hydrogenase indicated a definite relationship between species of *Chlorella*, *Scenedesmus*, and *Tetradismus* in the minimal haplotype network, and the identification of eight missing variants and the reflected evolutionary pattern were supported by the analysis of the enzyme's aa similarity in different species of microalgae (Fig. 1). Furthermore, the interspecific diversity we observed between species of microalgae suggests a divergent evolutionary strategy in the [FeFe]-hydrogenase enzyme, deriving in complementary functions –isofunctionalization and subfunctionalization– related to artificial selection pressure in some of the analyzed species (Peters et al. 2015).

Molecular hydrogen production and its relationship with the expression of *hyd* genes in *Chlorella vulgaris* and *Scenedesmus obliquus*

With the objective of knowing how the expression of the *hyd* genes correlates with the production of H₂ in *C. vulgaris* and *S. obliquus*, we analyzed their H₂ production and expression of *hyd* genes in conditions

inducing the activity of the hydrogenase enzyme.

The determination of H_2 in *Chlorella vulgaris* and *Scenedesmus obliquus* indicated some points to be highlighted (Table 1 and Fig. 4). When the microalgae were exposed to 1h of white light ($140 \mu E m^{-2} s^{-1}$), a production of 2 ± 0.2 mL H_2 /L and 1 ± 0.30 of mL H_2 /L was determined, in *Chlorella vulgaris* and *Scenedesmus obliquus*, respectively. The H_2 evolution kinetics of both microalgae were similar up to 5h of continuous exposure to white light, subsequently *Scenedesmus obliquus* showed better H_2 values compared to *Chlorella vulgaris*, during the following 19h. During the evaluated time course (24h), it was observed that *Scenedesmus obliquus* (16 ± 0.50 mL H_2 /L) is a better H_2 producer compared to *Chlorella vulgaris* (9.0 ± 0.40 mL H_2 /L) (Table 1 and Fig. 4a).

Table 1
Maximum productivity of H₂ in cultures of *C. vulgaris* and *S. obliquus* under conditions of light (140 $\mu\text{E m}^{-2} \text{s}^{-1}$)(data are shown as mean $\pm$ SD, n = 3).

Microalgae	Time (h)	Maximum production H ₂ (mL L ⁻¹)	Time (min)	Maximum production H ₂ (mL L ⁻¹)
<i>C. vulgaris</i>	1	2.0 ^a $\pm$ 0.20	30	1.0 ^a $\pm$ 0.20
	5	4.0 ^b $\pm$ 0.36	60	2.0 ^b $\pm$ 0.30
	9	6.0 ^c $\pm$ 0.24	90	2.0 ^b $\pm$ 0.25
	12	7.5 ^c $\pm$ 0.30	120	2.0 ^b $\pm$ 0.15
	16	8.5 ^d $\pm$ 0.28	150	2.0 ^b $\pm$ 0.20
	20	9.0 ^d $\pm$ 0.30	180	2.0 ^b $\pm$ 0.15
	24	9.0 ^d $\pm$ 0.40	210	3.0 ^c $\pm$ 0.17
			240	3.0 ^c $\pm$ 0.23
			270	3.0 ^c $\pm$ 0.33
			300	4.0 ^c $\pm$ 0.32
<i>S. obliquus</i>	1	2.5 ^A $\pm$ 0.40	30	2.0 ^A $\pm$ 0.25
	5	4.0 ^B $\pm$ 0.23	60	2.0 ^A $\pm$ 0.23
	9	8.0 ^C $\pm$ 0.33	90	2.0 ^A $\pm$ 0.27
	12	10 ^D $\pm$ 0.23	120	2.0 ^A $\pm$ 0.33
	16	13 ^E $\pm$ 0.32	150	2.0 ^A $\pm$ 0.24
	20	15 ^E $\pm$ 0.15	180	2.0 ^A $\pm$ 0.40
	24	16 ^E $\pm$ 0.50	210	2.0 ^A $\pm$ 0.32
			240	2.0 ^A $\pm$ 0.40
			270	4.0 ^B $\pm$ 0.52
			300	6.0 ^C $\pm$ 0.32

On the other hand, monitoring the evolution of H₂ during the first 5 h evaluated, with short intervals of 30 min (Fig. 4b), indicated that it is possible that H₂ is detected in both microalgae from the first 30 min under white light exposure (Table 1 and Fig. 4b). In *Chlorella vulgaris*, significantly different H₂ levels were detected after 180min (3h), while in *Scenedesmus obliquus* the H₂ values were significantly different up to 270 min (4.5h), indicating differences between the kinetics of both microalgae. Ruiz Marin et al. (2020) had previously argued that both algae presented variations in the duration of the lag phase before initiating H₂ production after being exposed to different light intensities, and suggested that the differences in the production of H₂ is because they have different ability to adapt quickly to the new culture conditions. The adaptation of other microalgae to new culture conditions could depend in part on regulation of the enzymes involved in H₂ production like HYD and FDX (Sun et al. 2013; Happe and Kaminski 2002). These variations in regulation will be dictated by the genome of each species analyzed.

Regulation of *hyd* genes in microalgae *Scenedesmus obliquus* and *Chlorella vulgaris*

The results of our analysis through qPCR of the relative expression of Hyd in the algae cultured in hydrogenase inducing media –made to know if the *hyd* gene is differentially regulated in *C. vulgaris* and *S. obliquus*– indicated that both strains showed higher levels of *hyd* expression at 1 h of culture (Fig. 5a). *C. vulgaris* showed 2.3 times more expression of the *hyd* gene than *S. obliquus*. Subsequently, in both strains, a decrease in the relative expression values was observed at 5 h of culture, until reaching a basal expression during the following 19 h.

It is known that the expression of the *hyd* genes is transient, and that their regulation can be inhibited by the products of photosynthesis (Weiner et al. 2018). Studies have shown that hydrogenase accumulates under dark anoxic adaptation conditions. Following such induction, exposure of algae to light supports high rates of H₂ production, but H₂ production ceases within a few minutes of illumination (Ghirardi 2015; Noone et al. 2017). This suggests that the observed increase in the expression of the *hyd* gene in *C. vulgaris* and *S. obliquus* within the first few hours after the transition from dark anoxia to light is the result of transcriptional regulation of the algal enzymes involved in this sudden change from dark to light, which takes place until the acclimatization of the algae and the activity of the hydrogenase becomes constant and basal (Fig. 5a).

With the aim of deepening the knowledge about regulation of *hyd* genes in the studied algae, we evaluated the relative expression of *hyd* genes in a 5 h period at 30 min intervals by qPCR. The results indicated higher expression levels at 30 min after the sudden change from darkness to light, reaching stable levels at 90 min (Fig. 5b).

This behavior pattern of *hyd* genes transcription in *C. vulgaris* and *S. obliquus* suggests that Hyd is involved in the production of H₂ in the first 90 min, suggesting that possibly after the first 90 min

hydrogenase is regulated by the levels of O_2 as a product of the bio-photolysis of H_2O that occurred during PSII in microalgae (Antal et al. 2003; Burlacot and Peltier 2018; Ghirardi et al. 2010). If we compare the levels of H_2 production during the first 30 min in both microalgae, we observe that it is not differential between algae (2 mL H_2/L) (Table 1 and Fig. 4b). Suggesting that the elevated expression levels in the first 30 minutes of light exposure (after a period of darkness) deregulates hydrogenase, enhancing its expression, later as the microalgae acclimatize to the new condition submitted, hydrogenase tends to regulate until to express sufficient levels to carry out its activity and contribute to the production of H_2 in both microalgae (Fig. 5b)

Discussion

The exploration of evolutionary trends in microalgae through sequence similarity data allows understanding and establishing families of genes and enzymes with biotechnological potential, such as [FeFe]-hydrogenase (Wang et al. 2020). However, the scarce information available on gene and enzyme sequences is a limiting factor for their potential application. It is of relevance to continue analyzing the coding genes for Hyd in algae in order to better understand the evolution and adaptation of enzymes in microalgae, and even to predict evolutionary trajectories of protein families of interest.

In this study we genetically characterize the *hyd* gene encoding hydrogenase in two microalgae (*C. vulgaris* and *S. obliquus*). According to the primary amino acid characteristics of the enzyme, we model dendrograms, construct a network of haplotypes and compare their three-dimensional structure among 11 genera of algae. The results indicated clustering variations (observed in the dendrogram and in the haplotype network) and in some algal genera the loss of aa residues responsible for the binding of the Iron/sulfur cluster “CPCACGCG” (*Coccomyxa subellipsoidea* (XP_005643907. 1) and *Tetrademus obliquus* (AA65921.1)) was observed. These differences found suggest the presence of isoforms or gene variants that encode hydrogenases in algae (Meuser et al. 2011; Engelbrecht et al. 2021).

Biotechnologically and biologically, there are important implications in finding different isoforms of [FeFe] hydrogenases in algae. The biotechnological importance lies in that the presence of isoforms of Hyd could impact on H_2 production by [FeFe] hydrogenases that reversibly and efficiently convert molecular hydrogen into protons and electrons, a catalytic activity for which the Iron/sulfur cluster is essential (Rodríguez-Maciá et al. 2018; Weiner et al. 2018). Group H of the active site of [FeFe] hydrogenases consist of a ([2Fe] ([2Fe] H) cluster covalently linked by cysteine to a canonical [4Fe-4S] cluster ([4Fe-4S] H). In *C. reinhardtii*, point mutations exchanging the cysteine residue that coordinates [4Fe-4S] H (Cys362) for histidine decreased the H_2 -production activity (Weiner et al. 2018). Also, in *C. reinhardtii*, a new [FeFe]-hydrogenase with an active site with selenium [2Fe2Se] was created by combining biological and chemical methods, which confers catalytic characteristics to the enzyme that lead to improved H_2 yields (Kertess et al. 2017). In that way, genetic modification of hydrogenases in a biotechnological context has allowed to increase H_2 yields in algae like *C. reinhardtii* (Meuser et al. 2012; Weiner et al. 2018) and *Chlorella* (Dubini and Ghirardi, 2015).

From the biological perspective, the presence of Hyd isoforms in algae has a relevance because the hydrogenase enzymes participate in a process that ensures their survival by participating in the vital process of photosynthesis (Petrova et al. 2020), it is therefore not surprising that these enzymes are highly conserved (Fig. 2, Fig. S2, Fig. S3). However, as it was demonstrated, small mutational changes could lead to the inactivation of genes (pseudogenization) or subject them to processes such as neofunctionalization, functionalization, and subfunctionalization, impacting on the process of photosynthesis and the production of H₂ in algae.

The differences in the grouping of hydrogenase enzymes in these algal genera that is observed in our dendrogram (Fig. 1) could reflect their evolution (D'Adamo et al. 2014; Meuser et al. 2011) as a result of the changes to which the genes encoding the enzymes have been subjected, which highlights the importance of research aimed at identifying these genetic changes in the enzyme. Unfortunately, few studies have been made of the phylogenetic relationships of enzymes involved in the production of H₂ in algae, and of how that relationship correlates with variations in H₂ productivity. However, the bioinformatic analysis of enzymes in algae has gained recent attention with the objective of increasing the yields of any product of interest (Kumar and Chowdhary 2016; Tamayo-Ordóñez et al. 2017). The study of hydrogenases within this context should be approached as a short term goal.

Regarding the production of H₂, both microalgae demonstrated to reach a maximum accumulation of H₂ at 24 h (*Scenedesmus obliquus* (16 ± 0.50 mL H₂/L) and *Chlorella vulgaris* (9.0 ± 0.40 mL H₂/L)) (Table 1 and Fig. 4a). The first point of detection of H₂ in both microalgae was at 30 min, which correlated with the highest levels of hydrogenase expression. It is observed that at the beginning of the kinetics both microalgae show similar trends in H₂ production, but after 5h under continuous light exposure, *S. obliquus* demonstrated to produce higher H₂ values compared to *Chlorella vulgaris* (Table 1 and Fig. 5a). The higher H₂ production values we observed in *C. vulgaris* compared to *S. obliquus* may depend on the presence in both algae of different genes coding for HYD and FDX, which results in differences in both enzymes' activity. In this context, Winker et al. (2002) identified in *Chlorella fusca* at least one *hyd* gene showing activity after anaerobic incubation. In *Chlorella vulgaris* BEIJ strain G-120, Touloupakis et al. (2020) reported a HYD activity of 25.5 ± 0.2 nmoles H₂ µg Chl⁻¹ h⁻¹ and a H₂ production value of 4.98 mL L⁻¹ h⁻¹ under light conditions, and of 2.08 mL L⁻¹ h⁻¹ in dark conditions, showing that, in this strain, HYD could be key to improve H₂ yields during the first stage of PSII in the presence of light.

The regulation of the *hyd* isoforms in the genus *Chlorella* has not been studied as extensively as in *Chlamydomonas reinhardtii* (Engelbrecht et al. 2021; Meuser et al. 2012; Sawyer and Winkler 2017). In some cases, the presence of *hyd* isoforms can be related to differences in H₂ production through the photosynthetic pathway. For example, Engelbrecht et al. (2017) found that the [FeFe]-hydrogenases CrHydA1 and CrHydA2 of *Chlamydomonas variabilis* NC64A had activities of 225 µmol H₂ mg⁻¹ min⁻¹ and 100 µmol H₂ mg⁻¹ min⁻¹ with PetF as an electron donor, and of 87 µmol H₂ mg⁻¹ min⁻¹ and 102

$\mu\text{mol H}_2 \text{ mg}^{-1} \text{ min}^{-1}$ with Fdx2, respectively. These results suggest that the affinity to electron donors of HYD isoforms depends on their encoding genes, which can be reflected in the production of H_2 .

On the other hand, Ruiz-Marin et al. (2020), reported that this same *Scenedesmus* strain turned out to be more H_2 producer compared to *Chlorella*, indicating that by stimulation with violet light, showed productivities of 128.0 mL H_2/L and 60.4 mL H_2/L , respectively. This author highlighted that both algae presented variations in time in the lag phase, as they were exposed to different intensities of light, to later initiate H_2 production, suggesting that the fact that the H_2 production kinetics in both microalgae differ after 5h under continuous white light exposure, it will depend on the ability of the microalgae to adapt quickly to the new culture condition. The adaptation of more microalgae to the new conditions could depend in part on the regulation of enzymes involved in the production of H_2 such as hydrogenases and ferredoxin (Antal et al. 2003; Burlacot and Peltier 2018; Ghirardi et al. 2010). These variations in regulation will be dictated by the genome of each species analyzed.

Also, Tinpranee et al. (2016) evaluated 13 isolates of *Chlorella* sp. and showed that the production of hydrogen could vary according to the anaerobic culture conditions (darkness/light), demonstrating maximum variations in H_2 productions in the range of 1 to 63 nmol $\text{H}_2 \text{ mg dry wt}^{-1}$ depending on the isolate analyzed. Also for *Chlorella* sp., Sun et al. (2011) reported H_2 production values of 1.5 to 4.5 mL/g VS for different inoculum–substrate ratios (ISRs) that were reached during the first 24 h of anaerobic fermentation. In *C. pyrenoidosa*, H_2 production values of 0.5 μmol have been reported at 24 h (Su et al. 2019), and in *C. vulgaris*, undetectable H_2 production values were reported during the first 24 h, but an H_2 production of 1.6 to 2.3 mmol per L (Lakaniemi et al. 2011) and ranges of H_2 production from 1.0 to 15 mL $\text{H}_2 \text{ g-VS}^{-1}$ at different substrate dosages were reached during the first 24 h of dark fermentation (Wieczorek et al. 2014).

According to the literature, it seems that the species in the genus *Chlorella* respond to different growing conditions adapting their H_2 production values accordingly. This same pattern was observed in the *Scenedesmus* genus. There is a report of *S. acuminatus* cultured in PLEM medium –with high concentrations of nitrogen (1890 mg L^{-1}), phosphorus (187 mg L^{-1}), potassium (1749 mg L^{-1}), and other micronutrients– achieving a H_2 production value of 2.1 nmol $\text{H}_2/\text{g chl/h}$ in cells grown for 21 h (Unpaprom et al. 2017). Also, in *Scenedesmus* sp. KMITL-O1 cultured in TAP medium under light and incubated under anaerobic conditions for 2 h, 1.4 nmol $\text{H}_2/\mu\text{g chl/h}$ were reached at 18 h (Rattana et al. 2012). *Scenedesmus obliquus* produced 42 pmol of H_2 when the strain was grown in TAP medium with cysteine (Marquez-Reyes et al. 2015).

Additionally, studies have been carried out in *S. obliquus* cultivated in media containing inductors or blockers of PSI and PSII, for example, *S. obliquus* produced 0.4 mL (mL PCV) $^{-1}$ of hydrogen when cultivated in the presence of 2,3-dichlorophenol for 24 h. The growth of *S. obliquus* during 5 days in the

presence of dichlorophenols resulted in H₂ yields between 4.7 mL (mL PCV)⁻¹ and 12.3 mL (mL PCV)⁻¹ (Papazi et al. 2012).

The reported differences in concentration of H₂ produced by *Chlorella* sp. and *Scenedesmus* sp. are the result of both variations in the conditions of the culture medium and the genetics of the strains used. Dark fermentative H₂ production could be influenced by various factors like inoculum, strain, reactor type, substrate, nitrogen, phosphate, metal ions, temperature, pH, etc. (Chen et al. 2011). The strain of algae used is a determining factor. According to the genetics of the strain, some algae could grow faster and accumulate higher biomass levels in less time, which could cause fast nutrients consumption and inhibit H₂ production. As mentioned above, our results of H₂ production in *C. vulgaris* are similar to those reported by Sun et al. (2011). In the case of *S. obliquus*, the values of H₂ production we obtained are different and higher than those reported in the literature, which suggests that the genetic background of this strain, associated with the genes encoding the enzymes involved in H₂ production, is an important factor for H₂ production.

In *C. reinhardtii* incubated for 1 h in the dark and after exposed to light (370 μEm⁻² s⁻¹) for 15 min, the activity of Hyd reached values of 2.3 μg HydA mg Chl⁻¹ during the first 2 minutes, which was related not to the increased production of H₂, but of oxygen (Weiner et al. 2018). Also, the accumulation of Hyd proteins in aerobically grown *C. reinhardtii* after the first 30 min of anaerobic adaptation in a solution with argon was also reported (Happe and Kaminski 2002). The differential expression of the genes coding for HydA1 and HydA2 was demonstrated by transcriptional analysis of *C. reinhardtii* cultures incubated in a sealed photobioreactor in sulfur-deprived TAP medium for 1–3 days, in which *hydA1* was more expressed than *hydA2*.

The differential behavior of *hyd* gene expression in *C. reinhardtii* was similarly observed by construction of a transcriptomic database in H₂ production conditions. The expression of two contigs, annotated as [FeFe]-hydrogenase HydA1 and HydA2, matched the H₂ evolution pattern, increasing during early H₂ production and decreasing when the H₂-production rate decreased, *hydA* being expressed up to 200 times more than *hydA2* (Yang et al. 2013). Similar results were reported through transcriptomic analysis of H₂ photoproduction in *C. pyrenoidosa* under nitrogen deprivation (Li et al. 2020), indicating that isoforms of the *hydA* genes also play an important role in the regulation of hydrogenase activities.

Our study explored the global expression of Hyd coding genes in *C. vulgaris* and *S. obliquus*. A more in-depth study involving the identification of isoforms would allow us to better understand how these enzymes are regulated, not only by knowing how Hyd isoforms are regulated in algae, but also by allowing us to make future genetic engineering studies to achieve the overexpression of Hyd for obtaining higher H₂ yields in algae (Yacoby et al. 2012).

It is important to highlight, that the search for alternative energy sources that do not involve fossil fuels has been a relevant goal that continues to be of importance today. Algae are organisms that have

demonstrated their ability to produce H₂ through photosynthesis involving PSI and PSII. Hydrogenase is a key enzyme with activity related to H₂ yields in algae. In this study, we show that *C. vulgaris* and *S. obliquus* produce 9.0 ± 0.40 mL H₂/L and 16 ± 0.50 mL H₂/L, respectively, after exposing to light cultures previously grown for 24 h in anaerobic conditions in darkness. Also, the results of our analysis of the relative expression of *hyd* genes showed their expression 30 min after exposing cultures to light. The differential behavior in the production of H₂ in the analyzed microalgae could be due to each microalgae's capacity to adapt to the culture conditions. In addition, the differential regulation of *hyd* isoforms in these microalgae could be key to *S. obliquus* being a better producer of H₂ in comparison to *C. vulgaris*. Deeper studies allowing us to identify all the Hyd isoforms in algae, would provide a better understanding of the evolution, regulation of transcription, and activity of this enzyme. Future studies of overexpression of this enzyme using different hosts could be relevant to increase H₂ yields in different organisms.

Declarations

• Ethics approval and consent to participate

Authors declare that they have no conflict of interest, financial or otherwise.

The authors declare to maintain the integrity of the investigation that:

The manuscript has not been submitted to more than one journal for simultaneous consideration.

The manuscript has not been published previously (partly or in full).

The single study is not split up into several parts to increase the quantity of submissions.

No data have been fabricated or manipulated (including images) to support our conclusions.

No data, text, or theories by others are presented as if they were the author's own ("plagiarism").

Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities - tacitly or explicitly - at the Autonomous University of Carmen, where the work has been carried out.

Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

• Consent for publication

Not applicable

• Availability of data and materials

All data generated or analysed during this study are included in this published article

• **Competing interests**

The authors declare that they have no competing interests Funding

• **Funding**

This work was financially supported by CONACYT with the scholarship provided to the Research Professor Yahaira de Jesus Tamayo Ordoñez with the CVU number 349678 during the National Postdoctoral Stay 1st Year 2019-1 and by the Research Professor Maria C. Tamayo Ordoñez (96377) with PRODEP project UACOAHPCTC-506 (Program for Teacher Development, Superior Type) and responsible for the Genetic Engineering Laboratory of the Department of Biotechnology of the Autonomous University of Coahuila

• **Authors' contributions**

YJTO: this researcher collaborated in the genetic analysis of hydrogenases in algae, specifically in the construction of haplotype networks by analyzing two regions of the protein. She got involved in the writing of the article

BAAG: this researcher performed the DNA extractions and from these, by means of PCR, amplified the 18SrDNA regions and hydrogenase genes. He also made the respective grouping dendograms between algal genera. He got involved in writing the article.

ARM: this researcher collaborated in the experimental design for the determination of H₂. Development of microalgae culture and sampling. He analyzed the database.

FATO: This researcher in the experimental design, statistical analysis of the database and design of figures.

IMMMD: This researcher determined the expression of H₂ under the selected conditions and data analysis

LJRG: This researcher analyzed the H₂ production data in both microalgae.

JARG: This researcher extracted the RNA from samples of the algal cultures

JCRH: This researcher performed the expression analysis of the hydrogenase genes evaluated through real time.

MCTO: This researcher was involved in the analysis of data obtained in this research, also financed the project and was involved in the writing of the article.

All authors read and approved the manuscript

• Acknowledgements

The authors express their gratitude to the Biotechnology Department of the Autonomous University of Coahuila for the infrastructure and facilities provided to carry out the research Project and UNACAR and CBG-IPN, for the facilities granted for the development of the project

• Authors' information (optional)

Not applicable

References

1. Antal TK, Krendeleva TE, Laurinavichene TV, Makarova VV, Ghirardi ML, Rubin AB, Tsygankov AA, Seibert M (2003) The dependence of algal H₂ production on Photosystem II and O₂ consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells. *BBA-Bioenergetics* 1607:153-160
2. Azwar MY, Hussain MA, Abdul-Wahab AK (2014) Development of biohydrogen production by photobiological, fermentation and electrochemical processes: a review. *Renew Sustain Energy Rev* 31:158-173
3. Bandelt HJ, Forster P, Röhl A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biol Evol* 16:37-48
4. Batista AP, Gouveia L, Marques PASS. (2018). Fermentative hydrogen production from microalgal biomass by a single strain of bacterium *Enterobacter aerogenes* – Effect of operational conditions and fermentation kinetics. *Renew Energy* 119:203-209
5. Batista AP, Moura P, Marques PASS, Ortigueira J, Alves L, Gouveia L (2014) *Scenedesmus obliquus* as feedstock for biohydrogen production by *Enterobacter aerogenes* and *Clostridium butyricum*. *Fuel* 117:537-543
6. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42:W252-W258
7. Burlacot A, Peltier G (2018) Photosynthetic electron transfer pathways during hydrogen photoproduction in green algae: mechanisms and limitations. In *Microalgal Hydrogen Production* (pp. 189-212)
8. Carrieri D, Wawrousek K, Eckert C, Yu J, Maness PC. (2011). The role of the bidirectional hydrogenase in cyanobacteria. *Bioresour Technol* 102:8368-837
9. Chien LF, Kuo TT, Liu BH, Lin HD, Feng TY, Huang CC (2012) Solar-to-bioH₂ production enhanced by homologous overexpression of hydrogenase in green alga *Chlorella* sp. DT. *Int J Hydrog Energy* 37:17738-17748

10. Chomczynski P, Mackey K, Drews R, Wilfinger W (1997) DNAzol®: a reagent for the rapid isolation of genomic DNA. *Biotechniques* 22:550-553
11. D'Adamo S, Jinkerson RE, Boyd ES, Brown SL, Baxter BK, Peters JW, Posewitz MC (2014) Evolutionary and biotechnological implications of robust hydrogenase activity in halophilic strains of *Tetraselmis*. *PloS one* 9:e85812
12. Derbyshire MK, Gonzales NR, Lu S, He J, Marchler GH, Wang Z, Marchler-Bauer A (2015) Improving the consistency of domain annotation within the Conserved Domain Database. *Database*. 2015: bav012
13. Díaz-Rey MR, Cortés-Reyes M, Herrera C, Larrubia MA, Amadeo N, Laborde M, Alemany LJ. (2015). Hydrogen-rich gas production from algae-biomass by low temperature catalytic gasification. *Catal Today*. 257:177-184
14. Dincer I, Acar C (2015) Review and evaluation of hydrogen production methods for better sustainability. *Int J Hydrogen Energy* 40:11094-11111
15. Dong M, Zhang X., Chi X, Mou S, Xu J, Xu D, Wang W, Ye N (2012) The validity of a reference gene is highly dependent on the experimental conditions in green alga *Ulva linza*. *Curr Genet* 58:13-20
16. Dubini A, Ghirardi ML (2015) Engineering photosynthetic organisms for the production of biohydrogen. *Photosynthesis Res* 123:241-253
17. Engelbrecht V, Liedtke K, Rutz A, Yadav S, Günzel A, Happe T (2021) One isoform for one task? The second hydrogenase of *Chlamydomonas reinhardtii* prefers hydrogen uptake. *Int J Hydrog Energy* 46:7165-7175
18. Eroglu E, Melis A (2011) Photobiological hydrogen production: recent advances and state of the art. *Bioresour Technol* 102:8403-8413
19. Fakhimi N, Gonzalez-Ballester D, Fernández E, Galván A, Dubini A (2020) Algae-Bacteria Consortia as a Strategy to Enhance H₂ Production. *Cells* 9:1353
20. Florin L, Tsokoglou A, Happe T (2001) A Novel Type of Iron Hydrogenase in the Green Alga *Scenedesmus obliquus* Is Linked to the Photosynthetic Electron Transport Chain. *J Biol Chem* 276:6125-6132
21. Forestier M, King P, Zhang L, Posewitz M, Schwarzer S, Happe T, Ghirardi M, Seibert M (2003) Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. *Eur J Biochem* 270:2750-2758
22. Ghirardi ML, Seibert M, Posewitz M, Wecker M (2010) Regulation of H₂ and CO₂ metabolism: Factors involved in partitioning of photosynthetic reductant in green algae. In 2010 Physical Biosciences Research Meeting (Vol. 38, p. 31)
23. Ghirardi ML. (2015). Implementation of photobiological H₂ production: The O₂ sensitivity of hydrogenases. *Photosynthesis Res*. 125: 383-393
24. Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can J Microbiol* 8:229-239

25. Happe T, Kaminski A (2002) Differential regulation of the Fe-hydrogenase during anaerobic adaptation in the green alga *Chlamydomonas reinhardtii*. Eur J Biochem 269:1022-1032
26. Jiménez-Llanos J, Ramírez-Carmona M, Rendón-Castrillón L, Ocampo-López C. (2020). Sustainable biohydrogen production by *Chlorella* sp. microalgae: A review. Int J Hydrogen Energy 45:8310-8328
27. Kamp C, Silakov A, Winkler M, Reijerse EJ, Lubitz W, Happe T (2008) Isolation and first EPR characterization of the [FeFe]-hydrogenases from green algae. BBA-Bioenergetics 1777:410-416
28. Kertess L, Wittkamp F, Sommer C, Esselborn J, Rüdiger O, Reijerse EJ, Apfel UP (2017) Chalcogenide substitution in the [2Fe] cluster of [FeFe]-hydrogenases conserves high enzymatic activity. Dalton Trans. 46:16947-16958
29. Khan S, Fu P (2020) Biotechnological perspectives on algae: a viable option for next generation biofuels. Curr Opin Biotechnol 62:146-152
30. Khetkorn W, Khanna N, Incharoensakdi A, Lindblad P (2013) Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. Biofuels 4:535-561.
31. Khetkorn W, Rastogi RP, Incharoensakdi A, Lindblad P, Madamwar D, Pandey A, Larroche C (2017) Microalgal hydrogen production—A review. Bioresour Technol 243:1194-1206
32. Kim DH, Kim MS (2011) Hydrogenases for biological hydrogen production. Bioresour Technol 102:8423-8431
33. Kumar GR, Chowdhary N (2016) Biotechnological and bioinformatics approaches for augmentation of biohydrogen production: A review. Renew Sust Energ Rev 56:1194-1206
34. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870-1874
35. Lakaniemi AM, Hulatt CJ, Thomas DN, Tuovinen OH, Puhakka JA (2011) Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. Biotechnol Biofuels 4:34-36
36. Lam MK, Loy ACM, Yusup S, Lee KT (2019) Biohydrogen production from algae. In *Biohydrogen* (pp. 219-245). Elsevier; 2019
37. Laurinavichene TV, Fedorov AS, Ghirardi ML, Seibert M, Tsygankov AA (2006) Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells. Int J Hydrog Energy 31:659-667
38. Li L, Zhang L, Gong F, Liu J (2020) Transcriptomic analysis of hydrogen photoproduction in *Chlorella pyrenoidosa* under nitrogen deprivation. Algal Res 47:101827
39. Limongi AR, Viviano E, Luca MD, Radice RP, Bianco G, Martelli G (2021) Biohydrogen from Microalgae: Production and Applications. Appl Sci 11:1616
40. Márquez-Reyes LA, del Pilar Sánchez-Saavedra M, Valdez-Vazquez I (2015) Improvement of hydrogen production by reduction of the photosynthetic oxygen in microalgae cultures of *Chlamydomonas gloeopara* and *Scenedesmus obliquus*. Int J Hydrog Energy 40:7291-7300

41. Melis A (2002) Green alga hydrogen production: progress, challenges and prospects. *Int J Hydrog Energy* 27:1217-1228
42. Meuser JE, Boyd ES, Ananyev G, Karns D, Radakovits R, Murthy UN, Posewitz MC (2011) Evolutionary significance of an algal gene encoding an [FeFe]-hydrogenase with F-domain homology and hydrogenase activity in *Chlorella variabilis* NC64A. *Planta* 234:829-843
43. Meuser JE, D'Adamo S, Jinkerson RE, Mus F, Yang W, Ghirardi ML, Posewitz M C (2012) Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: insight into the role of HYDA2 in H₂ production. *Biochem Biophys Res Commun* 417:704-709
44. Milrad Y, Schweitzer S, Feldman Y, Yacoby I (2018) Green algal hydrogenase activity is outcompeted by carbon fixation before inactivation by oxygen takes place. *Plant Physiol* 177:918-926
45. Mus F, Cournac L, Cardettini V, Caruana A, Peltier G (2005) Inhibitor studies on non-photochemical plastoquinone reduction and H₂ photoproduction in *Chlamydomonas reinhardtii*. *BBA-Bioenergetics* 1708:322-332.
46. Nagarajan D, Lee DJ, Kondo A, Chang JS (2017) Recent insights into biohydrogen production by microalgae—From biophotolysis to dark fermentation. *Bioresour Technol* 227:373-387
47. Nikolaidis P, Poullikkas A 2017 A comparative overview of hydrogen production processes. *Renew Sust Energ Rev* 67:597-611
48. Noone S, Ratcliff K, Davis R, Subramanian V, Meuser J, Posewitz MC, Ghirardi ML (2017) Expression of a clostridial [FeFe]-hydrogenase in *Chlamydomonas reinhardtii* prolongs photo-production of hydrogen from water splitting. *Algal Res* 22:116-121
49. Papazi A, Andronis E, Ioannidis NE, Chaniotakis N, Kotzabasis K (2012) High yields of hydrogen production induced by meta-substituted dichlorophenols biodegradation from the green alga *Scenedesmus obliquus*. *PLoS One* 7:e49037
50. Peters JW, Schut GJ, Boyd ES, Mulder DW, Shepard EM, Broderick JB, Adams MW (2015) [FeFe]-and [NiFe]-hydrogenase diversity, mechanism, and maturation. *BBA-Mol Cell Res* 1853:1350-1369
51. Petrova EV, Kukarskikh GP, Krendeleva TE, Antal TK (2020) The Mechanisms and Role of Photosynthetic Hydrogen Production by Green Microalgae. *Microbiology* 89:251-265
52. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45-e45.
53. Qian HF, Sheng GD, Liu W, Lu Y, Liu Z, Fu Z (2008) Inhibitory effects of atrazine on *Chlorella vulgaris* as assessed by real-time polymerase chain reaction. *Environ Toxicol Chem* 27:182-187
54. Rashid N, Lee K, Han JI, Gross M (2013) Hydrogen production by immobilized *Chlorella vulgaris*: optimizing pH, carbon source and light. *Bioproc Biosyst Eng* 36:867-872
55. Rattana S, Junyapoon S, Incharoensakdi A, Phunpruch S (2012) Hydrogen production by the green alga *Scenedesmus* sp. KMITL-O1 under heterotrophic conditions. The 8th International Symposium on Biocontrol and Biotechnology, Pattaya Thailand: 2012

56. Rochaix JD (2020) The dynamics of the photosynthetic apparatus in algae. In Photosynthesis in Algae: Biochemical and Physiological Mechanisms (pp. 57-82). Springer, Cham; 2020
57. Rodríguez-Maciá P, Kertess L, Burnik J, Birrell JA, Hofmann E, Lubitz W, Rüdiger O (2018) His-Ligation to the [4Fe–4S] Subcluster Tunes the Catalytic Bias of [FeFe] Hydrogenase. J Am Chem Soc 141:472-481
58. Ruiz-Marin A, Canedo-López Y, Chávez-Fuentes P (2020) Biohydrogen production by *Chlorella vulgaris* and *Scenedesmus obliquus* immobilized cultivated in artificial wastewater under different light quality. AMB Express 10:1-7
59. Ruiz-Marin A, Mendoza-Espinosa LG, Stephenson T (2010) Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour Technol 101:58-64
60. Santos-Merino M, Torrado A, Davis GA, Röttig A, Bibby TS, Kramer DM, Ducat DC (2021) Improved photosynthetic capacity and photosystem I oxidation via heterologous metabolism engineering in cyanobacteria. PNAS 118:e2021523118
61. Sawyer A, Winkler M (2017) Evolution of *Chlamydomonas reinhardtii* ferredoxins and their interactions with [FeFe]-hydrogenases. Photosynthesis Res 134:307-316
62. Sawyer A, Winkler M (2017) Evolution of *Chlamydomonas reinhardtii* ferredoxins and their interactions with [FeFe]-hydrogenases. Photosynth Res.134:307-316
63. Sharma S, Ghoshal SK (2015) Hydrogen the future transportation fuel: From production to applications. Renew Sust Energ Rev 43:1151-1158
64. Show KY, Yan Y, Ling M, Ye G, Li T, Lee DJ (2018) Hydrogen production from algal biomass—advances, challenges and prospects. Bioresour Technol 257:290-300
65. Srirangan K, Pyne ME, Chou CP (2011) Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. Bioresour Technol 102:8589-8604
66. Srivastava RK, Shetti NP, Reddy KR, Aminabhavi TM (2020) Biofuels, biodiesel and biohydrogen production using bioprocesses. A review. Environ Chem Lett 18:1049-1072
67. Staffell I, Scamman D, Abad AV, Balcombe P, Dodds PE, Ekins P, Shah N, Ward KR (2019) The role of hydrogen and fuel cells in the global energy system. Energy Environ Sci 12:463-491
68. Su D, Qi J, Liu X, Wang L, Zhang H, Xie H, Huang X (2019) Enzyme-Modulated Anaerobic Encapsulation of *Chlorella* Cells Allows Switching from O₂ to H₂ Production. Angew Chem 131:4032-4035
69. Sun J, Yuan X, Shi X, Chu C, Guo R, Kong H (2011) Fermentation of *Chlorella* sp. for anaerobic biohydrogen production: influences of inoculum–substrate ratio, volatile fatty acids and NADH. Bioresour Technol 102:10480-10485
70. Sun Y, Chen M, Yang H, Zhang J, Kuang T, Huang F (2013) Enhanced H₂ photoproduction by down-regulation of ferredoxin-NADP⁺ reductase (FNR) in the green alga *Chlamydomonas reinhardtii*. Int J Hydrog Energy 38:16029-16037

71. Swanson KD, Ratzloff MW, Mulder DW, Artz JH, Ghose S, Hoffman A, White S, Zadvorny OA, Broderick BB, King PW, Peters JW (2015) [FeFe]-hydrogenase oxygen inactivation is initiated at the H cluster 2Fe subcluster. *J Am Chem Soc* 137:1809-1816
72. Tamayo-Ordóñez MC, Rodríguez-Zapata LC, Narváez-Zapata JA, Tamayo-Ordóñez Y J, Ayil-Gutiérrez BA, Barredo-Pool F, Sánchez-Teyer LF (2016) Morphological features of different polyploids for adaptation and molecular characterization of CC-NBS-LRR and LEA gene families in *Agave* L. *J Plant Physiol* 195:80-94
73. Tamayo-Ordóñez YJ, Ayil-Gutiérrez BA, Sánchez-Teyer FL, De la Cruz-Arguijo EA, Tamayo-Ordóñez FA, Córdova-Quiroz AV, Tamayo-Ordóñez MC (2017) Advances in culture and genetic modification approaches to lipid biosynthesis for biofuel production and in silico analysis of enzymatic dominions in proteins related to lipid biosynthesis in algae. *Phycol Res* 65:14-28
74. Tamayo-Ordóñez YJ, Narvaez-Zapata JA, Sánchez-Teyer LF (2015) Comparative characterization of ribosomal DNA regions in different *Agave* accessions with economical importance. *Plant Mol Biol Rep* 33:2014-2029
75. Timmins M, Thomas-Hall SR, Darling A, Zhang E, Hankamer B, Marx UC, Schenk P M (2009) Phylogenetic and molecular analysis of hydrogen-producing green algae. *J Exp Bot* 60:1691-1702
76. Tinpranee N, Incharoensakdi A, Phunpruch S (2016) Hydrogen production by unicellular green alga *Chlorella* sp. LSD-W2 isolated from seawater in Thailand. *APST* 21:256-266
77. Unpaprom Y, Ramaraj R, Whangchai K (2017) A newly isolated green alga, *Scenedesmus acuminatus*, from Thailand with efficient hydrogen production. *Chiang Mai J Sci* 44:1270-1278
78. Wang Y, Yang H, Zhang X, Han F, Tu W, Yang W (2020) Microalgal Hydrogen Production. *Small Methods* 4:1900514
79. Weiner I, Shahar N, Feldman Y, Landman, S, Milrad Y, Ben-Zvi O, Avitan M, Dafni E, Schweitzer S, Eilenberg H, Atar S, Diamant A, Tuller T, Yacoby I. (2018) Overcoming the expression barrier of the ferredoxin-hydrogenase chimera in *Chlamydomonas reinhardtii* supports a linear increment in photosynthetic hydrogen output. *Algal Res* 33:310-315
80. Wieczorek N, Kucuker MA, Kuchta K (2014) Fermentative hydrogen and methane production from microalgal biomass (*Chlorella vulgaris*) in a two-stage combined process. *Appl Energy* 132:108-117
81. Winkler M, Heil B, Heil B, Happe T (2002) Isolation and molecular characterization of the [Fe]-hydrogenase from the unicellular green alga *Chlorella fusca*. *BBA-Gene Structure and Expression* 1576:330-334
82. Xia A, Cheng J, Lin R, Lu H, Zhou J, Cen K (2013) Comparison in dark hydrogen fermentation followed by photo hydrogen fermentation and methanogenesis between protein and carbohydrate compositions in *Nannochloropsis oceanica* biomass. *Bioresour Technol* 138:204-213
83. Xia A, Cheng J, Song W, Su H, Ding L, Lin R, Cen K (2015) Fermentative hydrogen production using algal biomass as feedstock. *Renew Sust Energy Rev* 51:209-230
84. Yacoby I, Tegler LT, Pochekailov S, Zhang S, King PW (2012) Optimized expression and purification for high-activity preparations of algal [FeFe]-hydrogenase. *PLoS One* 7:e35886

85. Yang S, Guarnieri MT, Smolinski S, Ghirardi M, Pienkos PT (2013) De novo transcriptomic analysis of hydrogen production in the green alga *Chlamydomonas moewusii* through RNA-Seq. *Biotechnol Biofuel* 6:118
86. Yang Z, Guo R, Xu X, Fan X, Li X (2011) Thermo-alkaline pretreatment of lipid-extracted microalgal biomass residues enhances hydrogen production. *J Chem Technol. Biotechnol* 86:454-460

Figures

Figure 1

Evolutionary relationship of [FeFe]-hydrogenase (H₂) in algae. Phylogeny was reconstructed by the neighbor-joining method (NJ) and cluster confidence was tested by 1000 bootstrap iterations. The amino acid sequences were aligned with Alignment Explorer/CLUSTALW program and the software Genetic and Molecular Evolution Analyses (MEGA version 6.0).

Figure 2

In silico tertiary structures of [FeFe]-hydrogenase (H₂) in algae. The prediction of tertiary conformation was performed using the SWISSMODEL program and *Chlamydomonas reinhardtii* was used as a model. Predicted three-dimensional structures of A) *Chlamydomonas reinhardtii*, B) *Chlorella vulgaris* (identified in this investigation), C) *Chlorella fusca*, D) *Chlorella sorokiniana*, E) *Chlorella Variabilis*, F) *Scenedesmus obliquus* (identified in this investigation) and G) *Scenedesmus* sp. indicated structural conformation of the enzyme. The black arrows indicate the iron / sulfur cluster, the blue arrows the chloride ion and the green arrows the arsenic, important for the functionality of the enzyme.

Figure 3

Mutational distance network of the enzyme [FeFe]-hydrogenase of microalgae. This analysis was performed with 120 amino acids from the (A) small subunit domain and (B) Carboxyl terminal of the [FeFe]-hydrogenase. The color of the circles represents the variants of [FeFe]-hydrogenase in different genera of microalgae. The line connecting the variants represents the mutational distance. V1CHLA: *Chlamydomonas reinhardtii* (XP_0011693376.1), V2NACH: *Nannochloropsis gaditana* (XP_005854541.1), V3CHLO: *Chlorella vulgaris*, V4SCEN: *Scenedesmus obliquus*, V5TETR: *Tetraselmis* sp. GSL018, V6CHLO: *Chlorella fusca* (CAC833290.1), V7CHLO: *Chlorella* sp. DT (ADK77883.1), V8TETR: *Tetraselmis obliquus* (CAC34419.1), V9CHLO: *Chlorella sorokiniana* (PRW60372.1), V10SCE: *Scenedesmus* sp. SEMC3 (AXU24207.1), V11RAP: *Raphidocelis subcapitata* (GBF94161.1), V12MON: *Monoraphidium neglectum* (XP_013906846.1), V13TET: *Tetraselmis obliquus* (AAG59621.1), V14TET: *Tetraspora* sp. CU2551 (AMY63159.1), V15CHL: *Chlorella variabilis* (AEA34989.1), V16NAN: *Nannochloropsis salina* (TFJ80951.1), V17VOL: *Volvox carteri* (XP_002948487.1).

Figure 4

Hydrogen production in *Chlorella vulgaris* and *Scenedesmus Obliquus*. The algal biomass was obtained by growth for 7 days and the algae were subsequently exposed under anaerobic conditions in sulfate free medium. The determination of molecular hydrogen production was by HPLC during the time intervals of 1, 5, 9, 12, 16, 20, and 24 hours (A) and 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min (B)

Figure 5

Expression relative of Hyd gene in *Chlorella vulgaris* and *Scenedesmus Obliquus*. Induction of hydrogenase was achieved by 24 h of dark incubation followed by immediate white light ($140 \mu\text{E m}^{-2} \text{s}^{-1}$) illumination of the reactors. Hyd expression relative were determined during the time intervals of 1, 5, 9, 12, 16, 20, and 24 hours (A) and 30, 60, 90, 120, 150, 180, 210, 240, 230, and 300 min of light exposure (B)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.tif](#)
- [TableS2.docx](#)
- [FigureS3.tif](#)
- [TableS1.docx](#)
- [figureS2.tif](#)