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Physiological and Environmental Factors Influencing Hydrogen Production by Unicellular Green Alga *Monoraphidium* sp. KMITL-1

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Abstract

With the growing global energy demand and the urgent need to reduce carbon emissions, hydrogen (H_2) has emerged as a promising clean energy carrier. Among various biological H_2 production, green algae present a sustainable and eco-friendly alternative due to their ability to produce H_2 via photobiological pathways. This study aimed to investigate H_2 production by unicellular green alga *Monoraphidium* sp. KMITL-1, isolated from hydroponic water at the Plant Tissue Culture Laboratory, King Mongkut's Institute of Technology Ladkrabang. The taxonomic identity of the strain, belonging to the genus *Monoraphidium* within the Selenastraceae family, was confirmed through morphological observation and molecular characterization using 23S plastid rRNA gene sequencing. Various physiological and environmental parameters influencing H_2 production were evaluated, including cell age, cell density, nutrient deprivation, carbon source, pH, temperature, and light intensity. A 24-hour-old culture with an OD_{750} of 0.8 exhibited a significant increase in H_2 production. The optimal medium was potassium-deprived Tris-acetate-phosphate (TAP-K) supplemented with glucose at a concentration of 350 mmol C-atom L^{-1}. The ideal environmental conditions for H_2 production were pH 7.2, a temperature of 30 °C, and a light intensity of 60 µmol photons m^{-2} s^{-1}. Under these optimized conditions, *Monoraphidium* sp. KMITL-1 achieved a maximum H_2 production rate of 67.976 ± 1.096 µmol H_2 mg Chl^{-1} h^{-1} and a cumulative H_2 yield of 3,190.436 ± 2.219 µmol H_2 mg Chl^{-1} after 72 h of incubation. These results highlight the potential of *Monoraphidium* sp. KMITL-1 for large-scale biohydrogen production and its applicability in the development of sustainable energy technologies.

Keywords: Hydrogen, Green algae, Nutrient deprivation, Carbon source, Hydrogenase activity

Introduction

Fossil fuels, the primary driver of global economic growth, have caused significant environmental damage and are projected to be depleted within the next 50 - 100 years [1]. As a result, the search for renewable, sustainable, and clean energy sources has become a top priority. Hydrogen (H_2) stands out as a promising alternative, offering a clean, carbon-free energy source with a high energy density (142 kJ g^{-1}) that can be efficiently converted into electricity and utilized for domestic and industrial applications [2]. H_2 can be generated through thermochemical, electrochemical, and biochemical processes. Among these, biological H_2 production using green microalgae has gained increasing attention due to its potential for sustainability and reliance on inexhaustible solar energy via photosynthesis.

Green algae are a highly diverse group of phototrophic eukaryotic organisms that mostly perform

oxygenic photosynthesis. They can produce H_2 using electrons and protons derived from the oxidation of water molecules during photosynthesis. Under anaerobic conditions, driven by sunlight, green algae can redirect their endogenous photosynthetic electron flow in the thylakoid membranes toward H_2 production [3]. This terminal reaction is catalyzed by the nuclear-encoded, chloroplast-localized [FeFe] hydrogenase enzyme [4,5]. H_2 production by green algae depends on several factors, including cell age and cell optical density [6], medium composition [7,8], temperature [9], pH [10], and light intensity [11].

Despite their potential, the overall efficiency of H_2 production in green algae remains low and species dependent. Key limitations include the extreme O_2 sensitivity of hydrogenase enzymes, competition for electron flow during photosynthesis, and suboptimal cultivation conditions [12,13]. Furthermore, most studies to date have focused on model organisms such as *Chlamydomonas reinhardtii*, with limited exploration of other algal taxa. The lack of systematic screening and strain development presents a significant bottleneck to the advancement of algal-based H_2 production technologies. Therefore, there is a pressing need to explore alternative green algal species that combine high H_2 productivity, stress tolerance, and compatibility with large-scale cultivation systems.

Monoraphidium sp., a planktonic freshwater microalga from the Selenastraceae family (Chlorophyceae, Chlorophyta), exhibits several traits desirable for industrial applications. It is known for its high biomass productivity, substantial lipid content, and adaptability to a range of environmental conditions [14-16]. This genus has been widely studied for applications in biodiesel production [17-21], wastewater treatment [20], and nutritional supplementation due to its rich content of protein, methionine, and α-linolenic acid [22]. Despite its promising biochemical profile, the H_2 production potential of *Monoraphidium* sp. remains largely unexplored. Preliminary studies have hinted at the H_2-producing capability of *Monoraphidium*, but comprehensive investigations are lacking. This knowledge gap underscores the need for detailed studies to assess its viability as a biohydrogen producer. Accordingly, this study focuses on *Monoraphidium* sp. KMITL-1, aiming to evaluate and optimize its H_2 production potential under varying culture conditions. The findings could contribute significantly to expanding the diversity of algal species utilized in sustainable H_2 production systems.

Materials and methods

Materials

The unicellular green alga *Monoraphidium* sp. KMITL-1 was isolated from hydroponic water at the Plant Tissue Culture Laboratory, Department of Biology, School of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The key materials used in the experimental procedures are listed in **Table 1**, and the overall experimental workflow is illustrated in **Figure 1**.

Table 1 Materials used in this study.

Materials	Manufacturers
Tris-acetate-phosphate (TAP) medium	
Acetic acid glacial	Carlo Erba (Cornaredo MI, Italy)
Ammonium chloride	Carlo Erba (Cornaredo MI, Italy)
Dipotassium hydrogen phosphate	Carlo Erba (Cornaredo MI, Italy)
Magnesium sulfate heptahydrate	Carlo Erba (Cornaredo MI, Italy)
Potassium dihydrogen phosphate	Carlo Erba (Cornaredo MI, Italy)
Tris(hydroxymethyl)aminomethane	Sigma-Aldrich (St. Louis, MO, USA)
Carbon sources	
Butanol	RCl Labscan™ (Ireland)
Citric acid	RCl Labscan™ (Ireland)
Ethanol	RCl Labscan™ (Ireland)

Materials	Manufacturers
Glucose	Biomark™ Laboratories (Pune, India)
Glycerol	Fisher Scientific (Loughborough, UK)
Lactose	Merck (Darmstadt, Germany)
Propanol	Fisher Scientific (Loughborough, UK)
Sucrose	Fisher Scientific (Loughborough, UK)
Hydrogenase activity	
Methyl viologen dichloride hydrate	Sigma-Aldrich (Singapore)
Sodium dithionite	Sigma-Aldrich (Singapore)
Triton X-100	Merck (Darmstadt, Germany)

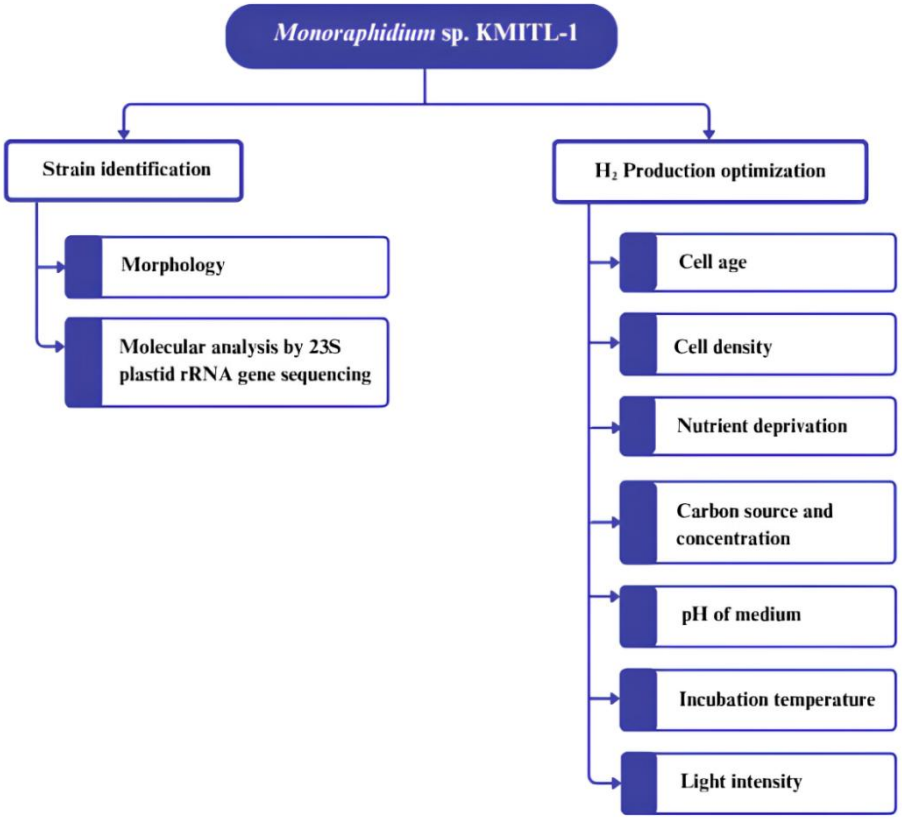

Figure 1 Flowchart illustrating the process of strain identification and optimization of H_2 production by the unicellular green alga *Monoraphidium* sp. KMITL-1.

Green algal cultivation and identification

Monoraphidium sp. KMITL-1 was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of TAP medium (pH 7.2) [23]. The culture was incubated on a rotary shaker at 120 rpm under continuous white light at an intensity of 30 µmol photons m^{-2} s^{-1} at 30 °C for 3 days. Morphological characteristics were examined using a bright-field microscope (Nikon Eclipse Ci-L, Japan). For molecular characterization, genomic DNA was extracted using the Wizard® SV DNA Purification System Kit (Promega, Madison, WI, USA). The 23S plastid rRNA gene was amplified by PCR Biometra® T Professional Thermocycler (Biometra, Göttingen, Germany) using the following primers: p23SrV_f1 (5'-GGACAGAAAGACCCTATGAA-3') and p23SrVr1 (5'-TCAGCCTGTTATCCCTAGAG-3') [24]. The 50 µL of PCR reaction contained 1X KAPA *Taq* Ready Mix (KAPA biosystems, South Africa), 25 pmol of each

primer, and 50 ng of total genomic DNA. PCR was performed under the following conditions: An initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min. The PCR product was purified using the QIAquick® PCR purification System Kit (Qiagen, Hilden, Germany) before sequencing. The 23S plastid rRNA gene sequence was analyzed for homology using the NCBI BLASTn tool [25]. Sequence alignment was performed using the ClustalW program [26], and molecular evolutionary genetic analysis was conducted using the Maximum Likelihood method with 1,000 bootstrap replicates, implemented in Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [27].

Growth measurement

Monoraphidium sp. KMITL-1 was cultivated in TAP medium under the previously described conditions for 5 days, with an initial optical density at 750 nm (OD_{750}) of approximately 0.1. The culture was sampled every 6 h to monitor growth. Growth was determined by measuring OD_{750} using a spectrophotometer (Shimadzu, UV-601, Japan). Total chlorophyll content was determined according to the method described by [28]. Cell concentration was measured using a hemocytometer (Boeco, Germany).

Measurement of H_2 production

Monoraphidium sp. KMITL-1 was cultivated in TAP medium for 48 h, harvested by centrifugation at 8,000× g at 4 °C for 10 min, and subsequently washed 3 times with fresh TAP medium. The cell suspension was adjusted to an OD_{750} of 0.5, and the cell pellet was resuspended in 5 mL of fresh TAP medium. The cell suspension was then transferred into a 12-mL glass vial, which was sealed with a rubber stopper and an aluminum crimp. To eliminate O_2 from the system, the vial was purged with argon gas for 10 min and subsequently shaken at 120 rpm under a light intensity of 30 μmol photons m^{-2} s^{-1}. H_2 production was analyzed using a gas chromatograph with a thermal conductivity detector (GC-TCD) (Hewlett-Packard, HP5890A GC, Japan), equipped with a molecular sieve 5 Å 60/80 mesh packed column, using 99.999% (v/v) argon gas as the carrier gas and 4% (v/v) H_2 in argon as the standard H_2 gas (Praxair, Thailand). The injector and detector temperatures were set at 100 °C, while the column temperature was maintained at 50 °C.

Measurement of hydrogenase activity

Hydrogenase (H_2ase) activity was measured in the presence of methyl viologen and sodium dithionite. A 2-mL reaction mixture contained 1 mL of algal cell suspension, 1 mL of 50 mM phosphate buffer solution (PBS) (pH 6.8) containing 10 mM methyl viologen, 40 mM sodium dithionite, and 0.2% (v/v) Triton X-100 [7]. The reaction was carried out at 30 °C in darkness for 30 min under anaerobic conditions. Hydrogenase activity, indicated by the amount of H_2 produced, was measured using a gas chromatograph.

Effect of cell age and cell density on H_2 production

Monoraphidium sp. KMITL-1 was cultivated in TAP for 24, 48, 72, and 96 h. Cells were subsequently harvested by centrifugation, washed, and resuspended in fresh TAP before measuring H_2 production. To investigate the effect of cell density on H_2 production, the cell suspension was adjusted to OD_{750} values of 0.2, 0.4, 0.6, 0.8, and 1.0 before measuring H_2 production by a gas chromatograph.

Effect of nutrient deprivation on H_2 production

Monoraphidium sp. KMITL-1 was cultivated in TAP for 24 h, harvested by centrifugation, washed, and resuspended in different types of TAP media, including potassium-deprived TAP (TAP-K), nitrogen-deprived TAP (TAP-N), phosphorus-deprived TAP (TAP-P), and sulfur-deprived TAP (TAP-S). Normal TAP served as the control. To remove sulfur in TAP-S, $MgSO_4.6H_2O$, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $CuSO_4.5H_2O$ were excluded from the medium, but the metal ions Mg^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} were replaced with $MgCl_2$, $FeCl_2$, $ZnCl_2$ and $CuCl_2$, respectively, at the same ion concentrations. In TAP-N, NH_4Cl was removed from the medium. In TAP-P, KH_2PO_4 and K_2HPO_4 were removed, but K^+ was replaced by adding KCl. In TAP-K, KH_2PO_4 and K_2HPO_4 were removed, but PO_4^{3-} was replaced by adding NaH_2PO_4 and Na_2HPO_4. The composition modifications for each TAP medium are summarized in **Table 2**. The cells were incubated under nutrient-deprived conditions for 48 h under light aerobic conditions. After incubation, the cells were harvested, resuspended in 5 mL of the respective media, and

transferred into a 12 mL gas vial before measurement of H_2 production and hydrogenase activity using a gas chromatograph.

Table 2 Composition of standard TAP medium and single-nutrient-deprived TAP media, including TAP-S, TAP-N, TAP-P, and TAP-K. The presence (+) and absence (−) symbols indicate the availability and unavailability of each chemical compound in the culture medium, respectively. Some chemical compounds were replaced with the indicated alternatives.

Composition	Type of medium				
	TAP	**TAP-S**	**TAP-N**	**TAP-P**	**TAP-K**
NH_4Cl	+	+	−	+	+
$MgSO_4.6H_2O$	+	$MgCl_2$	+	+	+
$FeSO_4.7H_2O$	+	$FeCl_2$	+	+	+
$ZnSO_4.7H_2O$	+	$ZnCl_2$	+	+	+
$CuSO_4.5H_2O$	+	$CuCl_2$	+	+	+
KH_2PO_4	+	+	+	KCl	NaH_2PO_4
K_2HPO_4	+	+	+	KCl	Na_2HPO_4

Effect of carbon source and concentration on H_2 production

Monoraphidium sp. KMITL-1 cells were harvested, washed, and resuspended in optimal nutrient-deprived TAP medium supplemented with different carbon sources, including glucose, lactose, ethanol, butanol, propanol, citric acid, and glycerol. Acetic acid served as the control carbon source. The corresponding carbon source concentrations were 17.5, 35, 70, 175, 350, 700 and 1,750 mmol C-atom L^{-1}, with the control condition set at 35 mmol C-atom L^{-1} (C-atom concentration in TAP). H_2 production was measured using a gas chromatograph.

Effect of pH, temperature, and light intensity on H_2 production

Monoraphidium sp. KMITL-1 cells were harvested, washed, and resuspended in optimized TAP medium under various conditions, including pH levels (6.0, 7.0, 7.2, 8.0, and 9.0), incubation temperatures (25, 30, 35, and 40 °C), and light intensities (0, 30, 60, 90, and 120 µmol photons m^{-2} s^{-1}). The control conditions of pH, temperature, and light intensity were 7.2, 30 °C, and 30 µmol photons m^{-2} s^{-1}, respectively. H_2 production was measured using a gas chromatograph.

Statistical analysis

All data are expressed as means of at least 3 independent experiments. Error bars represent the standard deviation. Significant differences were determined using one-way analysis of variance (ANOVA). The data were analyzed using Duncan's multiple range test in IBM SPSS Statistics version 29.0, with a significance level of $p < 0.05$.

Results and discussion

Microalgal identification

In this study, *Monoraphidium* sp. KMITL-1 was identified using both morphological and molecular analyses. Under light microscopy, it exhibited unicellular, green-coloured cells with a crescent or lunate shape, tapering at the ends. The cells measured 15 - 20 µm in length and 1 - 5 µm in width (**Figure 2(A)**). *Monoraphidium* is classified in the family Selenastraceae, and is characterized morphologically by cells containing a single nucleus, a parietal chloroplast without pyrenoids, and asexual reproduction via autospore formation [29]. The morphology of *Monoraphidium* sp. KMITL-1 closely resembled *Monoraphidium* sp. HDMA-11 [30] and showed similarities to various *Monoraphidium* strains previously reported [14,31]. Identification of

Monoraphidium species is primarily based on key morphological traits, including the shape of the cell apices (round, tapered, finger-like, or needle-like), overall cell shape (cylindrical, straight, slightly curved, reniform, arched, sigmoid, or spiral), and cell length [31]. Additionally, species can be distinguished by the presence or absence of a starch envelope and pyrenoid-penetrating thylakoids [14]. However, morphological characteristics alone are insufficient for precise genus identification; therefore, molecular characterization was employed to confirm the morphological classification.

For molecular analysis, the 23S plastid rRNA gene of *Monoraphidium* sp. KMITL-1 was sequenced and submitted to the NCBI GenBank under accession number PQ469933.1. Phylogenetic analysis revealed that *Monoraphidium* sp. KMITL-1 was closely related with 97.12% similarity to *Monoraphidium* sp. P5-4 and clustered with other *Monoraphidium* strains and green algae within the family Selenastraceae, such as *Ankistrodesmus*, *Selenastrum*, and *Raphidocelis* (**Figure 2(B)**). These strains were phylogenetically distinct from other genera such as *Scenedesmus*, *Tetradesmus*, *Chlorella*, *Chlamydomonas*, and *Chlorosarcinopsis* (**Figure 2(B)**). The molecular data confirmed that *Monoraphidium* sp. KMITL-1 belongs to the genus *Monoraphidium*. A previous study reported that *Monoraphidium* sp. P5-4, isolated from tropical freshwater, showed potential as a biodiesel feedstock [32]. However, to date, H_2 production by *Monoraphidium* species has not been much investigated.

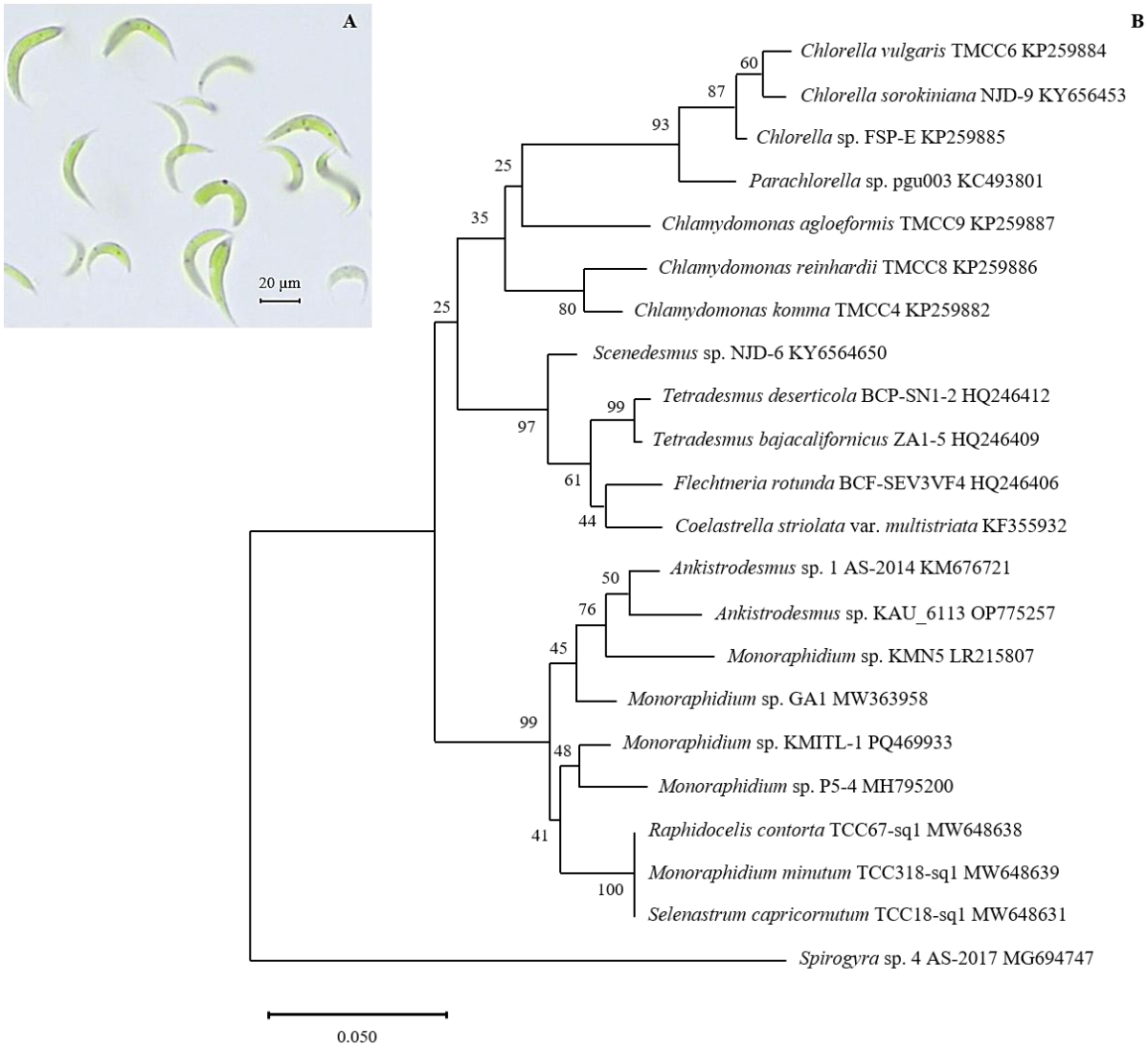

Figure 2 Observation using microscopy of *Monoraphidium* sp. KMITL-1 (A) and phylogenetic tree of *Monoraphidium* sp. KMITL-1 and other green microalgae constructed from the 23S plastid rRNA gene (B).

Growth of *Monoraphidium* sp. KMITL-1

Growth of *Monoraphidium* sp. KMITL-1 was determined by measuring OD_{750}, total chlorophyll content, and total cell concentration (**Figure 3**). The strain exhibited rapid growth, with a specific growth rate of 0.45 ± 0.02 day^{-1} and a doubling time of approximately 1.5 days. The highest total chlorophyll content of 9.67 ± 0.99 μg mL^{-1} and a maximum cell density of 7.44 ± 0.01×10^6 cells mL^{-1} were observed after 3 days of cultivation (**Figure 3**). This growth rate was approximately twice as high as that of *Monoraphidium neglectum* [33] and *Monoraphidium* sp. DeK19 [34]. The differences in growth rates are likely due to variations in cultivation media and algal species. In this study, *Monoraphidium* sp. KMITL-1 was grown in TAP medium under photoheterotrophic conditions using acetic acid as a carbon source, whereas *M. neglectum* was cultivated photoautotrophically in BG11 medium without an added carbon source, and *Monoraphidium* sp. DeK19 was grown in wastewater effluent [33,34]. These results suggest that TAP is a suitable medium for cultivating *Monoraphidium* species, as it provides essential nutrients along with various metals and minerals.

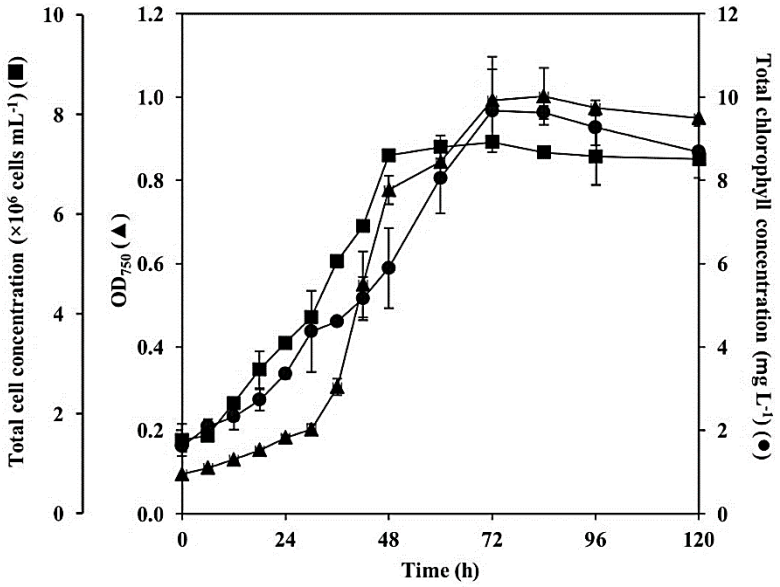

Figure 3 Growth of *Monoraphidium* sp. KMITL-1 cultivated in TAP by OD_{750} (▲), total chlorophyll concentration (●) and total cell concentration (■) measurements.

Effect of cell age and cell density on H_2 Production

The growth phases of algal cells, or cell ages, are associated with different metabolic activities, including H_2 production. In this study, cells harvested at 24, 48, 72, and 96 h corresponded to the lag, mid-log, late-log, and stationary phases, respectively. The results showed that *Monoraphidium* sp. KMITL-1 cells cultivated in TAP for 48 h exhibited the highest H_2 production rate of 2.990 ± 0.021 μmol H_2 mg Chl^{-1} h^{-1} (**Figure 4**). Cells harvested at earlier or later time points displayed significantly lower H_2 production rates. Moreover, the 48-hour-old cells achieved the highest cumulative H_2 production, reaching 110.163 ± 0.406 μmol H_2 mg Chl^{-1} after 96 h of anaerobic incubation (**Figure 4**). These findings indicate that the growth phase significantly affects H_2 production, with cells in the logarithmic phase yielding the highest levels. This may be attributed to the high metabolic activity and photosynthetic efficiency during the logarithmic phase, which generates a large number of electrons through water oxidation at Photosystem II (PSII). These electrons serve as substrates for the hydrogenase enzymes responsible for H_2 production. In contrast, *Chlamydomonas reinhardtii* UTEX 90 was reported to produce the highest H_2 levels during the late exponential phase [35].

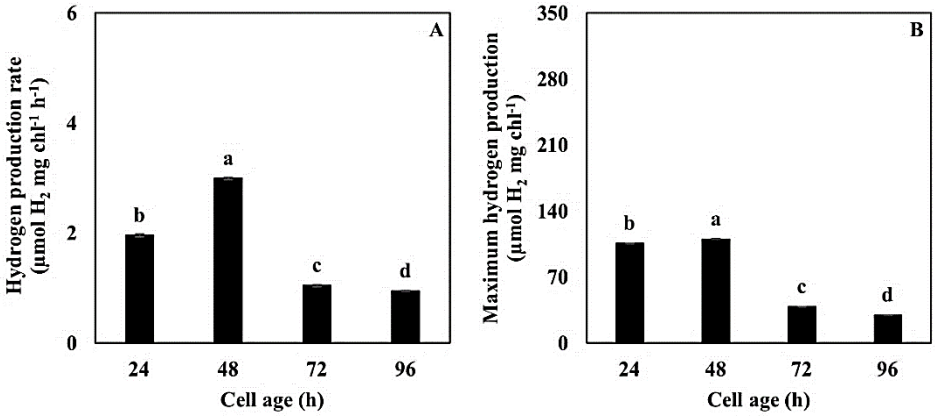

Figure 4 H_2 production rate (A) and maximum cumulative H_2 production (B) by *Monoraphidium* sp. KMITL-1 under different cell ages. Statistical analysis was performed using one-way ANOVA, with significance considered at $p < 0.05$. Data are presented as mean ± standard deviation.

The effect of cell density on H_2 production was investigated using 48-hour-old cultures of *Monoraphidium* sp. KMITL-1 at varying OD_{750} values. The results showed that increasing the OD_{750} from 0.2 to 0.8 led to higher H_2 production rates, corresponding with increased cell densities. Cultures with an OD_{750} of 0.8 exhibited the highest H_2 production rate of 4.803 ± 0.060 μmol H_2 mg Chl^{-1} h^{-1} and achieved the maximum cumulative H_2 production of 288.127 ± 1.197 μmol H_2 mg Chl^{-1} after anaerobic incubation for 48 h (**Figure 5**). This enhanced production at OD_{750} of 0.8 can be attributed to optimal PSII activity, resulting in greater electron availability for H_2ase-mediated H_2 production. At lower cell densities (OD_{750} less than 0.8), reduced PSII activity likely led to insufficient electron generation. Conversely, at OD_{750} values higher than 0.8, H_2 production decreased, probably due to limited light penetration within the culture vials, which hindered effective photosynthesis and electron generation. Similar findings have been reported in *Chlamydomonas reinhardtii* CC-125 and *Scenedesmus* sp. KMITL-OVG1 [8,36]. Based on these findings, *Monoraphidium* sp. KMITL-1 cultures at a cell age of 48 h and an OD_{750} of 0.8 were selected for subsequent studies on H_2 production.

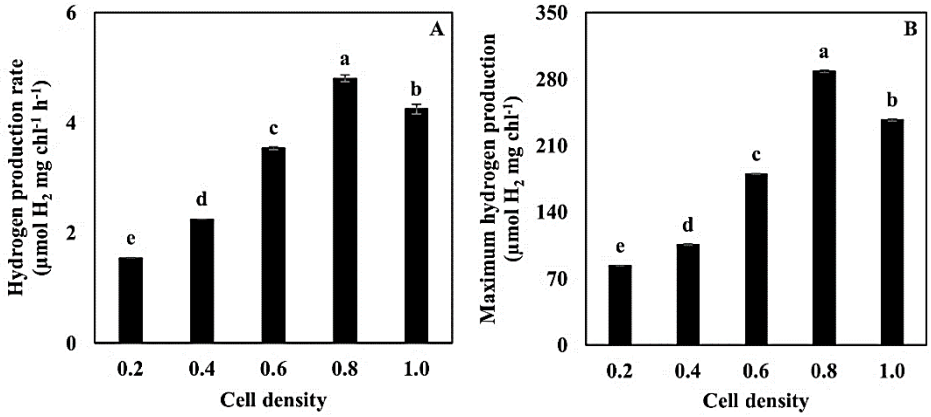

Figure 5 H_2 production rate (A) and maximum cumulative H_2 production (B) by *Monoraphidium* sp. KMITL-1 under different cell densities. Statistical analysis was performed using one-way ANOVA, with significance considered at $p < 0.05$. Data are presented as mean ± standard deviation.

Effect of nutrient deprivation on H_2 and O_2 production, and H_2ase activity

The H_2 and O_2 production rates, along with H_2ase activity, of *Monoraphidium* sp. KMITL-1 was investigated under various nutrient-deprived conditions. *Monoraphidium* sp. KMITL-1 cells exhibited the highest H_2 production rate of 11.388 ± 0.032 μmol H_2 mg Chl^{-1} h^{-1}, the lowest O_2 production rate of 4.455 ± 0.016 μmol O_2 mg Chl^{-1} h^{-1} and the highest H_2ase activity at 3.540 ± 0.026 μmol H_2 mg Chl^{-1} min^{-1} in cells incubated in TAP-K (**Table 3**). This H_2 production rate was approximately 2- to 5-fold higher than those observed under other nutrient deprivation conditions. Similarly, the maximum cumulative H_2 production yield of 677.102 ± 0.574 μmol H_2 mg Chl^{-1} was observed in cells incubated in TAP-K after 48 h of anaerobic incubation (**Table 3**). H_2 production by cells incubated in TAP-N, -P, and -S was significantly lower than that in normal TAP (**Table 3**). The lowest H_2 production rate of 2.426 ± 0.034 μmol H_2 g Chl^{-1} h^{-1}, was recorded in cells incubated in TAP-S (**Table 3**). In this study, H_2 production was inversely related to O_2 production but corresponded with H_2ase activity (**Table 3**). Since O_2 is a strong inhibitor of H_2ase, a decrease in O_2 levels resulted in higher H_2ase activity, ultimately enhancing H_2 production.

Table 3 H_2 production, O_2 production and H_2ase activity of *Monoraphidium* sp. KMITL-1 under different nutrient-deprived conditions. Data are presented as mean ± standard deviation.

Type of medium	H_2 production rate (μmol H_2 mg Chl^{-1} h^{-1})	Maximum H_2 production (μmol H_2 mg Chl^{-1})	O_2 production rate (μmol O_2 mg Chl^{-1} h^{-1})	Maximum O_2 production (μmol O_2 mg Chl^{-1})	H_2ase activity (μmol H_2 mg Chl^{-1} min^{-1})
TAP	4.885 ± 0.032[b]	287.859 ± 0.543[b]	5.992 ± 0.604[b]	403.421 ± 0.454[b]	1.167 ± 0.001[b]
TAP-K	11.388 ± 0.032[a]	677.102 ± 0.574[a]	4.455 ± 0.016[a]	264.923 ± 0.398[a]	3.540 ± 0.026[a]
TAP-N	4.061 ± 0.014[c]	265.163 ± 2.441[c]	7.448 ± 0.003[c]	545.140 ± 0.946[c]	0.817 ± 0.007[c]
TAP-P	3.626 ± 0.083[d]	136.356 ± 0.706[d]	8.052 ± 0.012[d]	605.741 ± 0.117[d]	0.755 ± 0.011[d]
TAP-S	2.426 ± 0.034[e]	100.135 ± 0.732[e]	9.539 ± 0.124[e]	691.163 ± 0.835[e]	0.536 ± 0.002[e]

Essential macronutrients, including S, N, P, and K, play critical roles in regulating the growth and metabolism of phototrophic microalgae. Deficiencies in these nutrients impose cellular stress, ultimately leading to the cessation of cell division and growth [37]. In this study, K deprivation was found to significantly enhance H_2 production. The lack of K impaired protein synthesis and decreased PSII activity, primarily by disrupting the replacement of the D1 protein [38]. As a result, the rate of O_2 evolution through PSII fell below the rate of respiratory O_2 consumption, creating anoxic conditions in closed systems. These anoxic conditions, in turn, induced H_2ase activity and promoted H_2 production [38]. In addition, the increased H_2 production might be due to the reduction of starch accumulation and the acceleration of starch degradation, leading to the provision of many electrons, the substrate for H_2ase [36]. Previous reports showed that K deprivation promoted H_2 production only in *Scenedesmus* strains [36,38]. This study shows that *Monoraphidium* is another green algal strain whose H_2 production is enhanced by K deprivation.

The deprivation of N, S, and P imposes severe stress on green algal growth, as these nutrients are essential components of proteins, nucleic acids, and other critical biomolecules. In *Monoraphidium* sp. KMITL-1, deprivation of N, P, or S did not enhance H_2 production. Among these nutrient deprivations, N deprivation appeared to have the least impact on H_2 production (**Table 3**). N deprivation has been reported to stimulate H_2 production in other algal species, such as *Chlamydomonas reinhardtii* [39], *Chlorella* sp. ChiW4 [40], and *Chlorella* sp. LSD-W2 [28], primarily through increased electron availability from photosynthesis and the degradation of accumulated starch. P deprivation has been shown to enhance H_2 production in marine green microalgae *Chlorella* sp., by promoting starch accumulation and facilitating anaerobic conditions [41].

In the present study, S deprivation led to the most significant reduction in both H_2ase activity and H_2

production, likely due to the lack of S required for H₂ase synthesis. Nevertheless, previous studies have demonstrated prolonged H_2 photoproduction under S deprivation in *C. reinhardtii* [42]. Under S-deprived conditions, the inability to resynthesize the D1 protein results in the gradual inhibition of PSII-dependent O_2-evolving activity, subsequently lowering O_2 levels, which can induce H₂ase activity and promote H_2 production. The decrease in H_2 production by *Monoraphidium* sp. KMITL-1 under N, P, and S deprivation requires further investigation. These findings suggest that the pattern of H_2 production differs depending on the algal strains. H_2 production observed in *Monoraphidium* sp. KMITL-1 closely resembles that of *Scenedesmus* species but differs from that of other green algal strains.

Effect of carbon source and concentration on H_2 production

H_2 production by *Monoraphidium* sp. KMITL-1 was evaluated in TAP-K supplemented with various carbon sources, including acetic acid, butanol, citric acid, glucose, glycerol, ethanol, lactose, propanol, and sucrose, each at an equivalent carbon atom concentration of 35 mmol C-atom L^{-1} (matching the carbon content of standard TAP medium). Among the tested carbon sources, glucose supplementation resulted in the highest H_2 production rate of 28.555 ± 0.093 µmol H_2 mg Chl^{-1} h^{-1}, with a cumulative H_2 yield of 1,446.683 ± 0.585 µmol H_2 mg Chl^{-1} after anaerobic incubation for 48 h (**Figure 6**). This yield was approximately 2.5-fold higher than that observed with acetic acid supplementation. Other carbon sources led to comparatively lower H_2 production rates and yields. The enhanced H_2 production with glucose is likely attributable to increased intracellular reducing equivalents generated via glycolysis, which facilitate electron transfer to H₂ase [43]. Therefore, glucose was selected for further investigation into the effects of carbon source concentration on H_2 production.

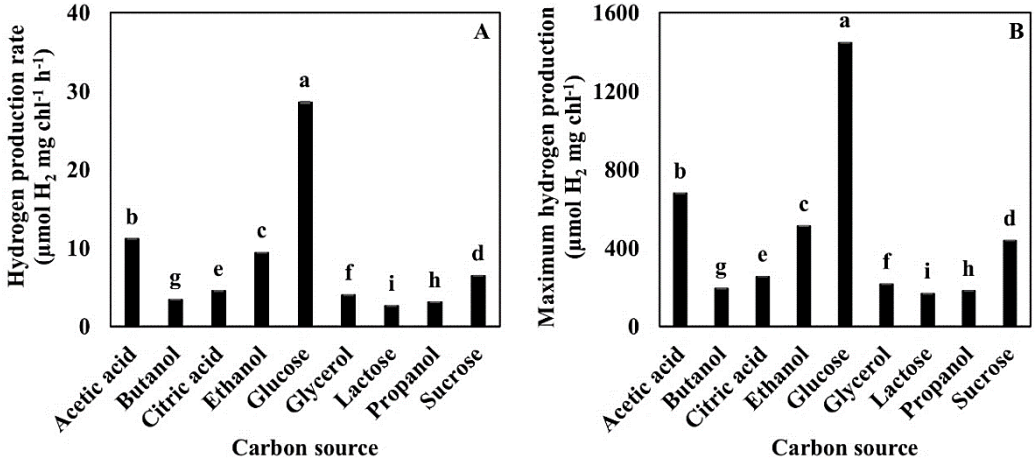

Figure 6 H_2 production rate (A) and maximum cumulative H_2 production (B) by *Monoraphidium* sp. KMITL-1 incubated in TAP-K medium supplemented with different carbon sources. Statistical analysis was performed using one-way ANOVA, with significance considered at $p < 0.05$. Data are presented as mean ± standard deviation.

By varying the glucose concentration from 17.5 to 1,750 mmol C-atom L^{-1}, the highest H_2 production rate of 45.211 ± 0.156 µmol H_2 mg Chl^{-1} h^{-1} was achieved at a glucose concentration of 350 mmol C-atom L^{-1} (**Table 4**). This rate represented approximately a fourfold increase compared to the K-deprived medium supplemented with 35 mmol C-atom L^{-1} glucose. Increasing glucose concentration likely enhanced the availability of electrons, the substrates for H₂ase activity, as glucose can be metabolized through glycolysis and subsequent fermentative pathways to generate reducing equivalents [44]. However, further increases in glucose concentration beyond 350 mmol C-atom L^{-1} led to a decline in both H_2 production rate and

cumulative yield (**Table 4**). This reduction may be attributed to the toxic effects of excess glucose, which can impose metabolic stress on cells by necessitating additional energy for glucose export [45,46]. Moreover, the low pH of the medium (pH 3.4) (**Table 4**) at higher glucose concentrations may have created an acidic environment that inhibited key enzymatic activities. Based on these findings, glucose supplementation at 350 mmol C-atom L^{-1} was selected for subsequent experiments.

Table 4 H_2 production rate and maximum cumulative H_2 production by *Monoraphidium* sp. KMITL-1 cultured in TAP-K medium supplemented with varying glucose concentrations. Data are presented as mean ± standard deviation.

Glucose (mmol C-atom L^{-1})	**H_2 production rate (μmol H_2 mg Chl^{-1} h^{-1})**	**Maximum H_2 production (μmol H_2 mg Chl^{-1})**	**Final pH**
17.5	9.314 ± 0.782^{g}	487.766 ± 0.248^{g}	4.15
35	29.071 ± 0.020^{d}	$1{,}452.814 \pm 0.113^{d}$	4.20
70	31.151 ± 0.108^{c}	$1{,}543.631 \pm 1.757^{c}$	4.25
175	35.754 ± 0.258^{b}	$2{,}352.372 \pm 1.249^{b}$	4.58
350	45.211 ± 0.156^{a}	$2{,}642.277 \pm 0.289^{a}$	3.71
700	18.228 ± 0.012^{e}	870.195 ± 0.175^{e}	3.41
1,750	7.614 ± 0.017^{h}	176.731 ± 0.243^{h}	3.47

Effect of pH, temperature and light intensity on H_2 production

H_2 production by *Monoraphidium* sp. KMITL-1 incubated in TAP-K supplemented with 350 mmol C-atom L^{-1} glucose was measured across a pH range of 6.0 to 9.0. A pH of 7.2, corresponding to the standard TAP medium, was used as the control. The maximum H_2 production rate of 45.506 ± 0.331 μmol H_2 mg Chl^{-1} h^{-1} was observed at pH 7.2 (**Figure 7(A)**). This rate was significantly higher than those recorded at other pH values. These results suggest that the H_2ase of *Monoraphidium* sp. KMITL-1 exhibits its highest catalytic activity for H_2 production at pH 7.2. Although lower pH conditions provide a higher concentration of protons, the substrate for hydrogenase, the enzyme activity was reduced under more acidic conditions, likely due to pH-induced enzyme inactivation. This result is consistent with previous studies found that *Chlorella* sp. KLSc59 and *C. reinhardtii* exhibited maximum H_2 production at pH 7.0 [6,47].

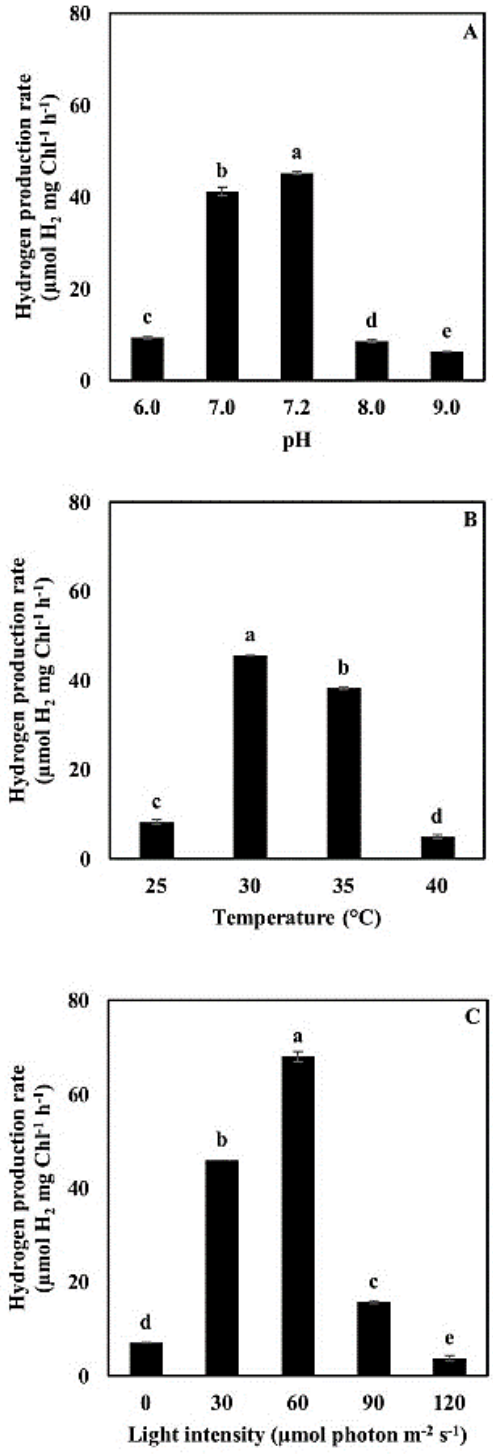

Figure 7 H_2 production rate of *Monoraphidium* sp. KMITL-1 under different initial pH values (A), temperatures (B), and light intensities (C). Statistical analysis was performed using one-way ANOVA, with significance considered at $p < 0.05$. Data are presented as mean ± standard deviation.

Among the tested incubation temperatures ranging from 25 to 40 °C, *Monoraphidium* sp. KMITL-1 showed the highest H_2 production rate of 45.585 ± 0.237 µmol H_2 mg Chl^{-1} h^{-1} at 30 °C (**Figure 7(B)**). Incubation at temperatures lower or higher than 30 °C led to a reduction in H_2 production. Similar to pH, the incubation temperature plays a critical role in regulating H_2ase activity, with 30 °C identified as the optimal

temperature for maximal H_2 production by *Monoraphidium* sp. KMITL-1. However, the optimal incubation temperature for H_2 production varies depending on the species of green algae. Under light intensities ranging from 0 - 120 µmol photon m^{-2} s^{-1}, *Monoraphidium* sp. KMITL-1 showed the highest H_2 production rate of 67.976 ± 1.096 µmol H_2 mg Chl^{-1} h^{-1} at 60 µmol photon m^{-2} s^{-1} (**Figure 7(C)**). This rate was approximately 10-fold and 1.5-fold higher than that observed under dark conditions and under a light intensity of 30 µmol photons m^{-2} s^{-1}, respectively. Furthermore, *Monoraphidium* sp. KMITL-1 achieved the maximum cumulative H_2 production of 3,190.436 ± 2.219 µmol H_2 mg Chl^{-1} after 72 h of incubation at 60 µmol photons m^{-2} s^{-1}. Light serves as an essential energy source for H_2 production through photosynthesis, which generates the electrons required by H_2ase. However, photosynthesis also produces oxygen, a strong inhibitor of hydrogenase activity. Therefore, an optimal light intensity is necessary to balance sufficient electron supply with minimal O_2 accumulation. These findings are consistent with a previous study in which a light intensity of 60 µmol photons m^{-2} s^{-1} resulted in the highest H_2 production in *Chlorella* sp. LSD-W2 [10]. In contrast, *Chlamydomonas reinhardtii* exhibited maximum H_2 production at a higher light intensity of 200 µmol photons m^{-2} s^{-1} [48].

Conclusions

This study demonstrated that physiological and environmental factors significantly influence H_2 production by the unicellular green alga *Monoraphidium* sp. KMITL-1. Key factors examined included cell age, cell density, nutrient deprivation, carbon source and concentration, pH, temperature, and light intensity. The optimal conditions for H_2 production were identified as a 48-hour-old culture with OD_{750} of 0.8. Potassium deprivation enhanced H_2 production by approximately 2.5-fold compared to non-deprived conditions. Among the carbon sources and concentrations tested, glucose at a concentration of 350 mmol C-atom L^{-1} significantly stimulated H_2 production. These findings confirm that *Monoraphidium* sp. KMITL-1 is a promising candidate for sustainable and efficient biohydrogen production. Its ability to produce H_2 under defined and controllable conditions highlights its potential for integration into renewable energy systems. Future studies should focus on optimization of large-scale cultivation systems, and investigation of long-term H_2 production stability under outdoor conditions. Moreover, co-cultivation with bacteria or use of waste-derived substrates may further improve the feasibility and economic viability of algal-based H_2 production systems.

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Declaration of generative AI in scientific writing

The authors acknowledge the use of the generative AI tool ChatGPT in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by the AI. The authors take full responsibility for the content and conclusions of this work.

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