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Combined Enhancement of Biohydrogen Production in the Halotolerant Cyanobacterium *Aphanothece halophytica* by Reducing Agents and Glucose Under Nitrogen-Deprived Dark Anaerobic Conditions

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Abstract

Hydrogen (H_2) is a promising renewable energy carrier because of its high energy density and environmentally friendly characteristics. The halotolerant cyanobacterium *Aphanothece halophytica* is a potential biological H_2 producer because of its ability to generate H_2 via oxygen-sensitive hydrogenase activity under nitrogen-deprived dark anaerobic conditions. This study investigated the effects of various chemical supplements on H_2 production by *A. halophytica*. Initial screening identified glucose, sodium dithionite, and dithiothreitol (DTT) as effective stimulants for enhancing H_2 production. Optimization experiments established 10 mM glucose, 5 mM DTT, and 1 mM sodium dithionite as the optimal concentrations for H_2 production. Combined supplementation under these optimized conditions resulted in the highest H_2 production rate of 0.588 ± 0.028 mL·L^{-1}·h^{-1} and maximum cumulative H_2 yield of 8.445 ± 0.750 mL·L^{-1} after incubation for 72 h, representing increases of approximately 170–180%, compared with the untreated control. Simultaneously, maximum cumulative O_2 production decreased from 20.176 ± 0.850 to 10.405 ± 0.480 mL·L^{-1}, corresponding to approximately a 50% reduction. These findings demonstrate that simultaneous optimization of electron supply, oxygen minimization, and intracellular redox balance using selected stimulants effectively enhances H_2 production by *A. halophytica*, providing a simple and practical strategy for improving biological H_2 production.

Keywords: biohydrogen; cyanobacteria; *Aphanothece halophytica*; glucose; sodium dithionite; dithiothreitol; nitrogen deprivation

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1. Introduction

Global energy consumption continues to increase due to population growth, industrial development, and expanding technological demands. The continued dependence on fossil fuels has contributed to greenhouse gas emissions, climate change, air pollution, and concerns regarding long-term energy security. Consequently, the development of renewable

and sustainable energy technologies has become a global research priority. Hydrogen (H_2) is considered a promising energy carrier because of its high gravimetric energy density (141.9 $MJ \cdot kg^{-1}$) and clean utilization. Its combustion with O_2 produces water as the main product without direct emissions of carbon dioxide, sulfur oxides, or particulate pollutants, while fuel cells can convert H_2 into electricity with high efficiency [1,2]. These characteristics have positioned H_2 as an important component of future low-carbon energy systems, with global demand expected to increase as countries pursue decarbonization and hydrogen-based energy strategies [3].

Despite these advantages, industrial H_2 production is currently dominated by thermochemical processes, particularly steam methane reforming and coal gasification, which rely heavily on fossil resources and are associated with substantial carbon emissions and energy consumption [3,4]. Biological H_2 production has therefore attracted increasing attention as a potentially more sustainable alternative because it can utilize renewable resources and operate under relatively mild conditions [5]. Biological H_2 production is generally classified into dark fermentation, photofermentation, direct biophotolysis, and indirect biophotolysis according to the microorganisms and metabolic pathways involved [4]. Among biological systems, cyanobacteria are particularly attractive because they can use sunlight, water, and carbon dioxide while generating H_2 through photosynthetic and fermentative metabolism. Cyanobacterial H_2 evolution is primarily mediated by hydrogenase and nitrogenase, which use cellular reducing equivalents to catalyze proton reduction [4,5]. However, the high O_2 sensitivity of these enzymes, especially hydrogenase, represents a major constraint on H_2 production. O_2 accumulation can inhibit hydrogenase activity and interfere with electron allocation toward H_2 evolution, thereby reducing overall productivity [6,7]. Controlling O_2 accumulation while maintaining sufficient electron supply to H_2-producing pathways is therefore an important strategy for improving cyanobacterial biohydrogen production.

Several approaches have been investigated to overcome these limitations, including optimization of cultivation conditions, nutrient manipulation, metabolic and genetic engineering, and supplementation with exogenous compounds [8]. Among these approaches, nitrogen deprivation is particularly effective because it induces metabolic reprogramming and promotes the mobilization of intracellular carbon reserves. Under nitrogen-limited conditions, cellular resources are redirected from biomass synthesis toward the degradation of stored carbohydrates, particularly glycogen, and the activation of fermentative metabolism [9,10]. This process generates reducing equivalents, such as NADH and reduced ferredoxin, which can contribute to electron transfer toward H_2-producing reactions [10]. Thus, improving the availability and allocation of reducing power represents an important avenue for increasing H_2 evolution under nitrogen-deprived conditions. In parallel, strategies that minimize O_2 accumulation can alleviate inhibition of hydrogenase and create more favorable conditions for H_2 production [11].

The halotolerant unicellular cyanobacterium *Aphanothece halophytica* is a promising candidate for biological H_2 production because of its ability to tolerate high salinity and osmotic stress. This physiological adaptability may facilitate cultivation in saline environments and reduce competition for freshwater resources [12]. Under nitrogen deprivation and dark anaerobic conditions, *A. halophytica* can mobilize intracellular glycogen, providing carbon substrates for glycolytic and oxidative pentose phosphate metabolism and generating reducing equivalents that support H_2 evolution [13]. Nevertheless, the efficiency of this process can be limited by the availability of suitable electron donors and the persistence of O_2 generated or retained within the system.

Previous work has shown that reducing O_2 accumulation can substantially improve H_2 production by *A. halophytica*. For example, co-cultivation with O_2-consuming bacteria enhanced H_2 production by alleviating O_2-mediated inhibition of hydrogenase activity [14].

This finding indicates that O_2 removal or reduction is an effective complementary strategy for improving H_2 evolution. Exogenous carbon sources may provide another means of increasing electron availability. Glucose, for instance, can be metabolized through central carbon metabolic pathways to generate reducing equivalents that may be utilized by H_2-producing enzymes [13]. Reducing agents may provide an additional benefit by decreasing O_2 availability and maintaining a more favorable chemical environment for anaerobic electron transfer [15–17]. Therefore, combining metabolic stimulation with O_2 mitigation may address two important constraints on H_2 production: limited electron availability and enzyme inhibition by O_2.

Although exogenous carbon sources and reducing agents have been investigated in biological H_2-producing systems, their effects on *A. halophytica* under nitrogen-deprived dark anaerobic conditions remain insufficiently characterized. Limited information is available regarding the relative effectiveness of different classes of exogenous stimulants and their effects on H_2 and O_2 production in this halotolerant cyanobacterium. Compounds with different physiological functions, including sugars, reducing agents, phytohormones, vitamins, and mineral supplements, may influence H_2 production through complementary effects on carbon metabolism, electron transfer, enzyme activity, or cellular stress responses. A systematic evaluation of these compounds could therefore identify effective strategies for improving H_2 production beyond the optimization of individual cultivation parameters.

Accordingly, this study investigated the effects of selected exogenous stimulants, including sugars, reducing agents, phytohormones, vitamins, and mineral supplements, on H_2 production by *A. halophytica* under nitrogen-deprived dark anaerobic conditions. The most effective stimulants were identified based on their effects on H_2 production, while changes in O_2 production were also evaluated to assess their potential contribution to process performance. The findings provide insight into the metabolic and redox factors influencing biohydrogen production in *A. halophytica* and may contribute to the development of more efficient biological H_2 production strategies using saline-adapted cyanobacteria.

2. Materials and Methods

2.1. Growth and Adaptation Conditions

The experimental procedure consisted of three sequential stages: (1) cell growth under nutrient-replete light aerobic conditions, (2) nitrogen-deprived adaptation under light aerobic conditions, and (3) H_2 production under nitrogen-deprived dark anaerobic conditions. First, the halotolerant cyanobacterium *A. halophytica*, at an optical density at 730 nm (OD_{730}) of 0.1, was cultivated in 250 mL Erlenmeyer flasks containing 100 mL of BG11 medium (pH 7.4) [18] supplemented with Turk Island salt solution [19]. The cultures were incubated at 30 °C on a rotary shaker at 120 rpm under continuous illumination at a light intensity of 30 µmol photons $m^{-2} \cdot s^{-1}$ for 7 days. For the adaptation process, cells were harvested by centrifugation at 8000× *g* for 5 min at 4 °C and subsequently resuspended in 100 mL of nitrogen-deprived BG11 medium ($BG11_0$) supplemented with Turk Island salt solution. The cultures were maintained under shaking conditions for 2 days to initiate cellular responses associated with nitrogen deprivation before the H_2 production experiments. All experiments were performed using three independent biological replicates.

2.2. Measurement of H_2 and O_2 Production

Seven-day-old *A. halophytica* cultures adapted for 2 days in $BG11_0$ medium supplemented with Turk Island salt solution were harvested by centrifugation and resuspended in fresh $BG11_0$ medium to a final cell density corresponding to an OD_{730} of 20. A 5 mL aliquot of the cell suspension was transferred into a 12 mL glass vial and sealed with a rubber septum. Anaerobic conditions were established by purging the cell suspension

with argon gas for 15 min. The sealed vials were subsequently incubated in the dark at 30 °C with continuous shaking at 120 rpm for 120 h. H_2 and O_2 concentrations in the vial headspace were analyzed by gas chromatography (Hewlett-Packard HP5890A, Avondale, PA, USA) at 30 °C and 101.325 kPa [20]. High-purity argon (99.999% v/v) was used as the carrier gas at a constant flow rate of 20 mL·min^{-1}. The injector, column, and detector temperatures were maintained at 100, 50, and 100 °C, respectively. The instrument was calibrated using a certified standard gas mixture containing 4% (v/v) H_2 in argon (Praxair, Bangkok, Thailand). Gas concentrations were calculated from the corresponding GC measurements using the calibration relationship established with the certified standard. The amounts of H_2 and O_2 were calculated from their measured concentrations and the vial headspace volume, normalized to the culture volume, and expressed as mL·L^{-1}. Cumulative H_2 and O_2 production was determined at each sampling time point relative to the initial measurement. Production rates were calculated from the change in cumulative gas production over the corresponding incubation period and expressed as mL·L^{-1}·h^{-1}. The maximum H_2 production rate was calculated from cumulative H_2 production during the first 24 h of incubation, whereas maximum cumulative H_2 and O_2 yields were determined at their respective optimal sampling time points and expressed as mL·L^{-1}.

For comparison with previous studies, dry cell weight was determined from the difference between filter weights before and after cell collection. H_2 production was also normalized to dry biomass and expressed as μmol H_2 g^{-1} dry weight h^{-1} for the production rate and μmol H_2 g^{-1} dry weight for cumulative H_2 yield. Time-course measurements were used to characterize the temporal pattern of H_2 accumulation throughout the 120 h incubation period. All experiments were conducted using three independent biological replicates.

2.3. In Vivo Bidirectional Hydrogenase Activity Assay

In vivo bidirectional hydrogenase activity was determined by measuring H_2 production mediated by dithionite-reduced methyl viologen, as previously described [13]. Briefly, cells from each treatment were harvested by centrifugation and washed with 25 mM sodium phosphate buffer (pH 7.6) to remove residual culture medium and externally supplied compounds. Subsequently, 1 mL of cyanobacterial culture was mixed with 1 mL of indicated sodium phosphate buffer containing 20 mM sodium dithionite and 5 mM methyl viologen. The reaction mixture was transferred to a sealed serum vial, flushed with argon gas for 5 min to establish anaerobic conditions, and incubated in the dark at 30 °C for 30 min. The amount of H_2 produced was subsequently determined by gas chromatography equipped with a thermal conductivity detector (GC-TCD). Bidirectional hydrogenase activity was expressed as mL H_2 L^{-1}·min^{-1}.

2.4. Screening of Stimulants for Enhanced H_2 Production

To identify compounds capable of enhancing H_2 production by *A. halophytica*, stimulants belonging to five functional groups, including sugars, reducing agents, phytohormones, vitamins, and minerals, were evaluated. The sugar group consisted of glucose (Carlo Erba Reagents, Milan, Italy) and sucrose (Carlo Erba, Milan, Italy). The reducing agent group included sodium hydrosulfite (Sigma-Aldrich, Taufkirchen, Germany), DL-dithiothreitol (DTT) (Sigma-Aldrich, Oakville, ON, Canada), methyl viologen dichloride hydrate (Sigma-Aldrich, Taufkirchen, Germany), and L-cysteine (Sigma-Aldrich, Shanghai, China). The phytohormone group consisted of 6-benzylaminopurine (cytokinin) (Sigma-Aldrich, Taufkirchen, Germany), gibberellic acid (GA_3) (Sigma-Aldrich, St. Louis, MO, USA), indole-3-acetic acid (IAA) (Merck KGaA, Darmstadt, Germany), and phytomelatonin (Sigma-Aldrich, St. Louis, MO, USA). The vitamin group included cobalamin and

riboflavin (Sigma-Aldrich, St. Louis, MO, USA), as well as L-ascorbic acid (Vetec, Shanghai, China). The mineral group consisted of cobalt (II) chloride hexahydrate (Sigma-Aldrich, St. Louis, MO, USA), nickel (II) sulfate hexahydrate (Sigma-Aldrich, St. Louis, MO, USA), ferric citrate (Sigma-Aldrich, St. Louis, MO, USA), magnesium sulfate heptahydrate (Merck KGaA, Darmstadt, Germany), and copper (II) sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO, USA). Each stimulant was individually added to nitrogen-adapted *A. halophytica* cultures at a final concentration of 1 mM. H_2 and O_2 concentrations in the vial headspace were determined using GC–TCD. Compounds exhibiting the greatest enhancement of H_2 production were subsequently selected for concentration optimization experiments.

2.5. H_2 Production Kinetic Analysis by Modified Gompertz Model

Cumulative H_2 production kinetics were analyzed using the modified Gompertz model by nonlinear regression [21]:

$$H = A \cdot e^{-e^{\left[\frac{R_m \cdot e}{A}(\lambda - t) + 1\right]}}$$

where H is the cumulative H_2 production ($mL \cdot L^{-1}$) at time t (h), A is the maximum cumulative H_2 production potential ($mL \cdot L^{-1}$), R_m is the maximum H_2 production rate ($mL \cdot L^{-1} \cdot h^{-1}$), λ is the lag phase (h), and e is Euler's number (2.71828). Model parameters were estimated by nonlinear least-squares regression using GraphPad Prism 11.2, and the fitted curves were compared with the experimental data.

2.6. Statistical Analysis

All experiments were performed in three independent biological replicates (n = 3). Data are presented as mean ± standard deviation (SD). The assumptions of normality of residuals and homogeneity of variance were assessed before one-way ANOVA. Statistical significance among treatment groups was evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$. Detailed ANOVA results, including degrees of freedom, F statistics, p-values, and partial eta squared values, are provided in Tables S1–S6.

3. Results

3.1. Screening of Stimulants for H_2 and O_2 Production

The effects of various stimulants belonging to five stimulant groups, including sugars, reducing agents, phytohormones, vitamins, and minerals, on H_2 and O_2 production by *A. halophytica* under nitrogen-deprived dark anaerobic conditions are summarized in Table 1. Among the sugar treatments, glucose exhibited the strongest stimulatory effect on H_2 production. Glucose supplementation increased the H_2 production rate from 0.218 ± 0.012 to 0.326 ± 0.018 $mL \cdot L^{-1} \cdot h^{-1}$ (approximately 50% increase) and increased maximum cumulative H_2 production from 3.043 ± 0.120 to 5.199 ± 0.180 $mL \cdot L^{-1}$ (approximately 71% increase). Simultaneously, maximum cumulative O_2 production decreased from 20.175 ± 0.450 to 14.503 ± 0.340 $mL \cdot L^{-1}$. In contrast, sucrose reduced cumulative H_2 production to 2.467 ± 0.110 $mL \cdot L^{-1}$ and increased cumulative O_2 accumulation to 24.008 ± 0.520 $mL \cdot L^{-1}$. Among the reducing agents tested, sodium dithionite and dithiothreitol produced the most pronounced positive effects. Sodium dithionite increased maximum cumulative H_2 production to 4.529 ± 0.180 $mL \cdot L^{-1}$ (approximately 49% increase), whereas DTT increased maximum cumulative H_2 production to 4.085 ± 0.160 $mL \cdot L^{-1}$ (approximately 34% increase). Both treatments were accompanied by lower O_2 accumulation compared with the control culture. In contrast, cysteine and methyl viologen at 1 mM decreased H_2 production compared with the untreated control.

Table 1. Screening of H_2 and O_2 production by *A. halophytica* incubated in $BG11_0$ medium supplemented with Turks Island salt solution and various stimulants at a final concentration of 1 mM under dark anaerobic conditions for 120 h. Different superscript letters within the same column indicate significant differences among all treatments according to Duncan's multiple range test at $p < 0.05$.

Chemical Supplements	Treatment	Maximum H_2 Production Rate ($mL \cdot L^{-1} \cdot h^{-1}$)	Maximum O_2 Production Rate ($mL \cdot L^{-1} \cdot h^{-1}$)	Maximum Cumulative H_2 Yield ($mL \cdot L^{-1}$)	Maximum Cumulative O_2 Yield ($mL \cdot L^{-1}$)
Control	-	0.218 ± 0.012^{c}	4.214 ± 0.085^{c}	3.043 ± 0.120^{d}	20.175 ± 0.450^{c}
Sugar	Glucose	0.326 ± 0.018^{a}	2.449 ± 0.062^{a}	5.199 ± 0.180^{a}	14.503 ± 0.340^{a}
	Sucrose	0.093 ± 0.008^{gh}	4.796 ± 0.105^{d}	2.467 ± 0.110^{f}	24.008 ± 0.520^{d}
Reducing agent	Cysteine	0.090 ± 0.008^{h}	5.334 ± 0.120^{e}	0.651 ± 0.050^{l}	34.865 ± 0.820^{i}
	Dithiothreitol	0.304 ± 0.015^{b}	2.829 ± 0.068^{b}	4.085 ± 0.160^{c}	18.047 ± 0.420^{b}
	Methyl viologen	0.185 ± 0.012^{d}	4.619 ± 0.105^{d}	1.894 ± 0.090^{hi}	26.046 ± 0.620^{ef}
	Sodium dithionite	0.310 ± 0.016^{b}	2.617 ± 0.061^{ab}	4.529 ± 0.180^{b}	15.715 ± 0.380^{a}
Phytohormone	Cytokinin	0.127 ± 0.010^{f}	6.034 ± 0.135^{f}	2.684 ± 0.110^{e}	25.385 ± 0.580^{e}
	Gibberellic acid	0.045 ± 0.006^{i}	6.793 ± 0.150^{h}	1.778 ± 0.080^{i}	32.064 ± 0.720^{h}
	Indole-3-acetic acid	0.109 ± 0.009^{g}	6.161 ± 0.138^{f}	2.503 ± 0.100^{f}	27.204 ± 0.620^{f}
	Phytomelatonin	0.099 ± 0.008^{gh}	6.701 ± 0.148^{h}	2.151 ± 0.090^{g}	29.481 ± 0.680^{g}
Vitamin	Cobalamin	0.185 ± 0.012^{d}	5.907 ± 0.130^{f}	2.056 ± 0.090^{gh}	36.275 ± 0.850^{j}
	Riboflavin	0.161 ± 0.011^{e}	7.246 ± 0.165^{i}	1.332 ± 0.070^{j}	51.274 ± 1.180^{m}
	L-Ascorbic acid	0.061 ± 0.006^{i}	9.645 ± 0.220^{k}	0.511 ± 0.040^{l}	63.389 ± 1.450^{n}
Mineral	Cobalt (II) chloride	0.161 ± 0.011^{e}	6.145 ± 0.140^{f}	2.414 ± 0.100^{f}	25.151 ± 0.580^{de}
	Nickel sulfate	0.152 ± 0.010^{e}	6.428 ± 0.145^{g}	0.975 ± 0.060^{k}	29.743 ± 0.680^{g}
	Ferric citrate	0.082 ± 0.007^{h}	8.007 ± 0.180^{j}	0.218 ± 0.030^{m}	35.119 ± 0.800^{ij}
	Magnesium sulfate	0.012 ± 0.002^{j}	10.746 ± 0.250^{l}	0.095 ± 0.015^{m}	39.715 ± 0.920^{k}
	Copper (II) sulfate	0.011 ± 0.002^{j}	10.862 ± 0.260^{l}	0.092 ± 0.015^{m}	43.701 ± 1.020^{l}

Phytohormones generally exerted inhibitory effects on H_2 production. Gibberellic acid produced the strongest inhibition, reducing the H_2 production rate to 0.045 ± 0.006 $mL \cdot L^{-1} \cdot h^{-1}$. Similarly, cytokinin, indole-3-acetic acid, and phytomelatonin all decreased H_2 production relative to the untreated control. Vitamin supplementation did not improve H_2 production. Riboflavin and L-ascorbic acid markedly increased O_2 accumulation while simultaneously reducing H_2 production. Likewise, mineral supplementation failed to enhance H_2 production. Ferric citrate, magnesium sulfate, and copper sulfate strongly inhibited H_2 evolution, with maximum cumulative H_2 yields below 0.25 $mL \cdot L^{-1}$. Notably, magnesium sulfate and copper sulfate reduced maximum cumulative H_2 production to only 0.095 ± 0.015 and 0.092 ± 0.015 $mL \cdot L^{-1}$, respectively. Overall, treatments that enhanced H_2 production generally reduced O_2 accumulation, whereas treatments associated with elevated O_2 production exhibited lower H_2 productivity. Based on these results, glucose, sodium dithionite, and DTT were selected for subsequent optimization experiments because they significantly enhanced H_2 production while simultaneously lowering O_2 accumulation relative to the control treatment.

3.2. Effect of Glucose Concentration on H_2 and O_2 Production

H_2 production increased progressively with increasing glucose concentration from 0.1 to 10 mM. The maximum H_2 production rate increased from 0.222 ± 0.008 $mL \cdot L^{-1} \cdot h^{-1}$ at 0.1 mM glucose to 0.371 ± 0.015 $mL \cdot L^{-1} \cdot h^{-1}$ at 10 mM glucose (approximately 67% increase) (Figure 1). Similarly, maximum cumulative H_2 production increased steadily and reached a maximum value of 6.394 ± 0.502 $mL \cdot L^{-1}$ at 10 mM glucose. This value was approximately 110% higher than that observed in the untreated control culture (3.043 ± 0.120 $mL \cdot L^{-1}$). The enhancement of H_2 production was accompanied

by a substantial reduction in O_2 production. The O_2 production rate decreased from 4.214 ± 0.120 mL·L^{-1}·h^{-1} in the control culture to 1.729 ± 0.065 mL·L^{-1}·h^{-1} at 10 mM glucose (Figure 1), corresponding to a reduction of approximately 59%. Concurrently, the highest H_2 production rate was observed at this concentration. However, higher glucose concentrations than 10 mM increased the O_2 production rate, resulting in a decrease in the H_2 production rate (Figure 1). In contrast, glucose concentrations above the optimum (20 and 50 mM) resulted in a pronounced decline in H_2 production accompanied by a marked increase in O_2 production. These findings suggest that excessive glucose supplementation adversely affected H_2-producing conditions, leading to substantially lower H_2 production than that observed at the optimal concentration of 10 mM.

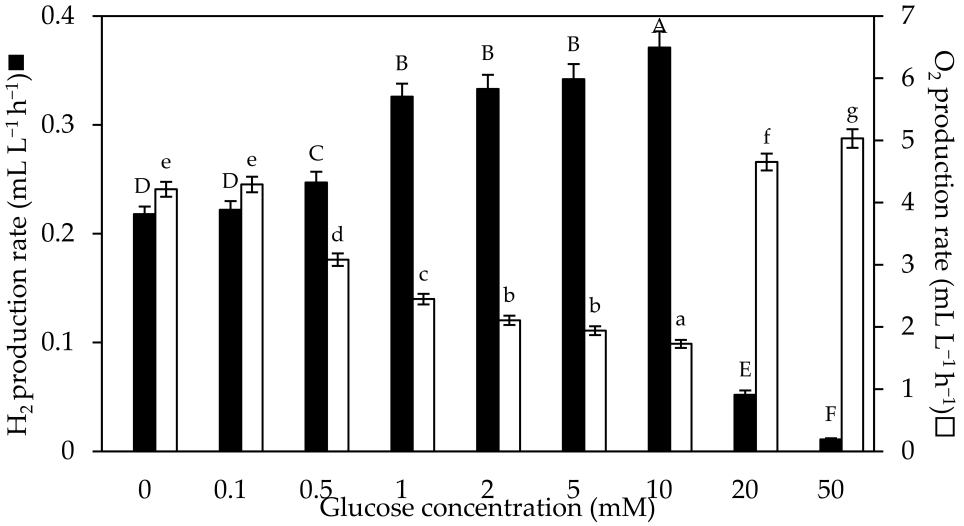


Figure 1. H_2 and O_2 production rates by *A. halophytica* incubated in $BG11_0$ medium supplemented with Turks Island salt solution containing different glucose concentrations under dark anaerobic incubation for 24 h. Values are presented as mean ± SD (n = 3). Error bars represent standard deviations from three independent biological replicates. Different uppercase letters indicate significant differences in H_2 production rates, whereas different lowercase letters indicate significant differences in O_2 production rates according to Duncan's multiple range test ($p < 0.05$).

3.3. Effect of Sodium Dithionite Concentration on H_2 and O_2 Production

H_2 production increased as sodium dithionite concentration increased from 0.1 to 1 mM. The highest H_2 production rate of 0.310 ± 0.014 mL·L^{-1}·h^{-1} was obtained at 1 mM sodium dithionite (Figure 2). This rate represented an increase of approximately 42% compared with the control. Concurrently, O_2 accumulation decreased with increasing sodium dithionite concentration. The lowest O_2 production rate was observed at 1 mM sodium dithionite, reaching 2.617 ± 0.075 mL·L^{-1}·h^{-1}, compared with 4.214 ± 0.120 mL·L^{-1}·h^{-1} in the untreated control (Figure 2). Further increases in sodium dithionite concentration beyond 1 mM resulted in decreased H_2 production. At 10 mM sodium dithionite, the H_2 production rate declined to 0.111 ± 0.006 mL·L^{-1}·h^{-1}. Simultaneously, the O_2 production rate increased to 4.672 ± 0.135 mL·L^{-1}·h^{-1} (Figure 2). These findings indicate that moderate O_2 scavenging promotes H_2 evolution, whereas excessive reducing conditions adversely affect H_2-producing metabolism.

3.4. Effect of DTT Concentration on H_2 and O_2 Production

H_2 production increased progressively as DTT concentration increased from 0.1 to 5 mM. Maximum H_2 production rates were observed at 1–5 mM DTT, with no significant differences among these concentrations (Figure 3), approximately a 41% increase compared with the control. However, 5 mM DTT produced the highest cumulative H_2

yield at 4.502 ± 0.360 mL·L^{-1} after dark anaerobic incubation for 72 h and was therefore selected as the optimal concentration for subsequent experiments. The increase in H_2 production was accompanied by a reduction in O_2 production. The lowest O_2 production rate was observed at 5 mM DTT, reaching 2.646 ± 0.055 mL·L^{-1}·h^{-1}, compared with 4.214 ± 0.085 mL·L^{-1}·h^{-1} in the untreated control (Figure 3). These results indicate that improvement of the cellular redox environment positively influences H_2-producing metabolism. However, further increases in DTT concentration resulted in reduced H_2 production. At 10 mM DTT, the H_2 production rate decreased to 0.148 ± 0.007 mL·L^{-1}·h^{-1}, while the O_2 production rate increased to 4.95 ± 0.095 mL·L^{-1}·h^{-1} (Figure 3).

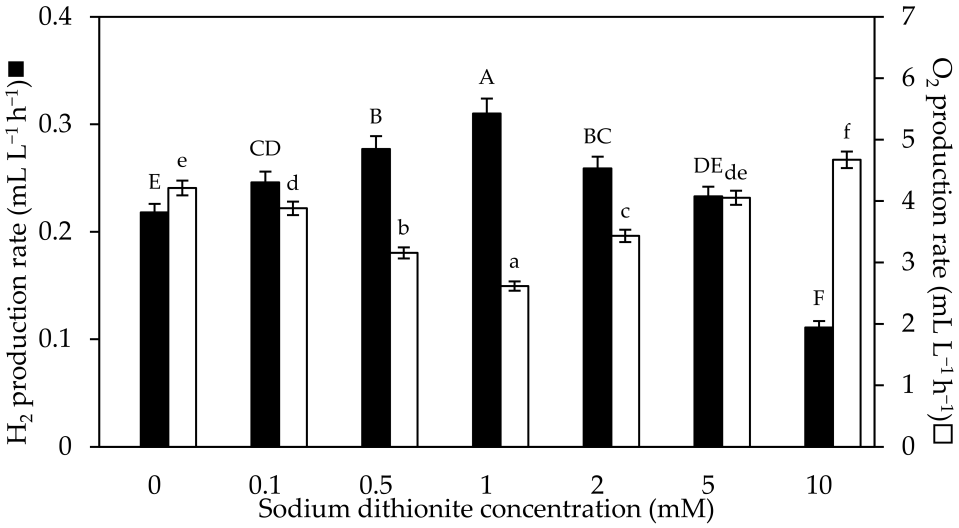


Figure 2. H_2 and O_2 production rates by *A. halophytica* incubated in $BG11_0$ medium supplemented with Turks Island salt solution containing different concentrations of sodium dithionite under dark anaerobic incubation for 24 h. Values are presented as mean ± SD (n = 3). Error bars represent standard deviations from three independent biological replicates. Different uppercase letters indicate significant differences in H_2 production rates, whereas different lowercase letters indicate significant differences in O_2 production rates according to Duncan's multiple range test ($p < 0.05$).

3.5. Combined Effects of Combined Stimulants with Optimal Concentrations on H_2 Production and Hydrogenase Activity

The effects of the single and combined selected stimulants with their optimal concentrations on H_2 production by *A. halophytica* are presented in Figure 4. Among all treatments evaluated, the combined supplementation of 10 mM glucose, 1 mM sodium dithionite, and 5 mM DTT resulted in the greatest enhancement of H_2 production. The combined treatment achieved the highest H_2 production rate of 0.588 ± 0.028 mL·L^{-1}·h^{-1}, which represented an increase of approximately 170% compared with the control. This rate was significantly higher than that obtained with single supplementation of glucose with 0.371 ± 0.018 mL·L^{-1}·h^{-1}, sodium dithionite with 0.311 ± 0.016 mL·L^{-1}·h^{-1}, DTT with 0.307 ± 0.015 mL·L^{-1}·h^{-1}, and the untreated control with 0.218 ± 0.012 mL·L^{-1}·h^{-1} (Figure 4). Similarly, the maximum cumulative H_2 yield reached 8.445 ± 0.750 mL·L^{-1} after 72 h of incubation, representing approximately 178% enhancement compared with the control treatment of 3.043 ± 0.120 mL·L^{-1}. Among the individual stimulants, glucose produced the strongest positive effect on H_2 production, followed by sodium dithionite and DTT. These results indicated that the combined treatment significantly outperformed all individual stimulant treatments, indicating the existence of combined interactions among the three compounds. The time-course profiles demonstrated that the measured H_2 accumulation increased progressively during the first 72 h of incubation, followed by an

apparent plateau from 72 to 120 h across all treatments, indicating that H_2 production approached its maximum production potential during the later stage of incubation.

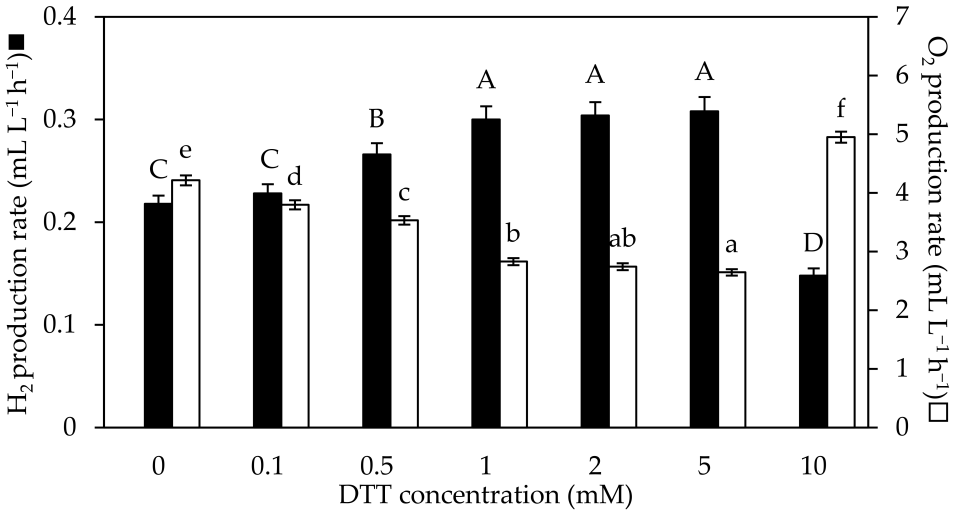


Figure 3. H_2 and O_2 production rates by *A. halophytica* incubated in $BG11_0$ medium supplemented with Turks Island salt solution containing different concentrations of DTT under dark anaerobic incubation for 24 h. Values are presented as mean ± SD (n = 3). Error bars represent standard deviations from three independent biological replicates. independent experiments. Different uppercase letters indicate significant differences in H_2 production rates, whereas different lowercase letters indicate significant differences in O_2 production rates according to Duncan's multiple range test ($p < 0.05$).

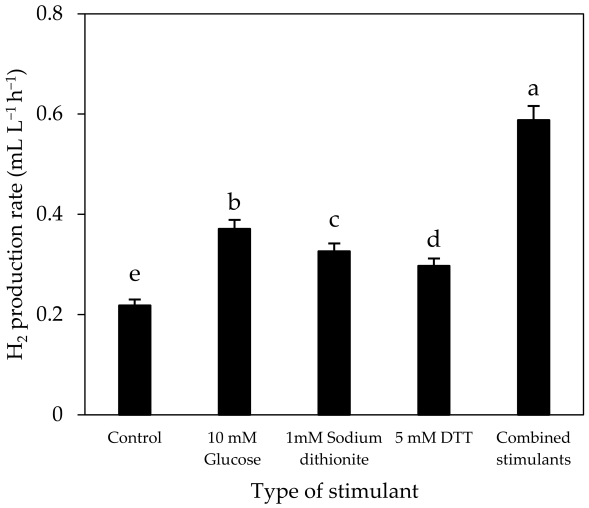


Figure 4. Effects of individual and combined stimulants at optimal concentrations on H_2 production by *A. halophytica* under nitrogen-deprived dark anaerobic conditions. Different letters indicate significant differences among treatments ($p < 0.05$). Values are expressed as mean ± SD (n = 3). Error bars represent standard deviations.

Time course profiles of cumulative H_2 production by *A. halophytica* treated with individual and combined stimulants were analyzed using the modified Gompertz model. All experimental profiles were fitted to the model to characterize the H_2 production kinetics under the different treatments (Figure 5). The estimated maximum H_2 production potential (A) was 3.271, 6.859, 4.994, 5.004, and 9.132 mL·L^{-1} for the control, 10 mM glucose, 1 mM sodium dithionite, 5 mM DTT, and combined treatment, respectively. Similarly, the estimated maximum H_2 production rate (Rm) increased from 0.064 mL·L^{-1}·h^{-1} in the control to 0.143, 0.089, 0.089, and 0.181 mL·L^{-1}·h^{-1} under the respective treatments. The lag phase (λ) ranged from 16.24 to 19.91 h. Notably, the experimentally measured cumulative

H_2 production at 120 h corresponded to 94.8–97.1% of the respective A values, whereas 90.0–93.2% of the modeled production potential had already been reached by 72 h. Only a relatively small increase in cumulative H_2 production was observed from 72 to 120 h, indicating that H_2 accumulation had approached a plateau after approximately 72 h.

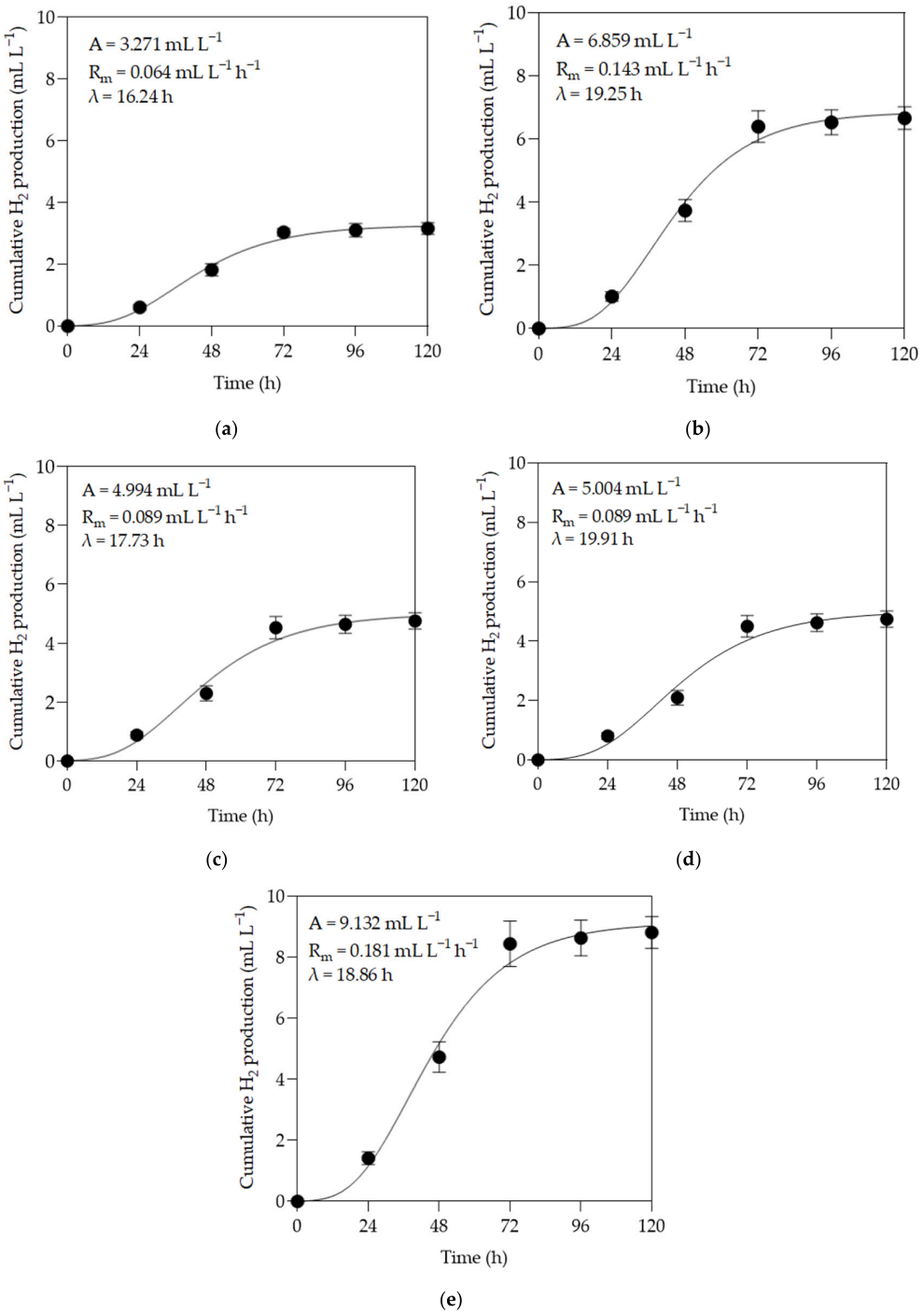


Figure 5. Time-course profiles of cumulative H_2 production fitted to the modified Gompertz model under different H_2-enhancement treatments: Control (**a**), 10 mM glucose (**b**), 1 mM sodium dithionite (**c**), 5 mM DTT (**d**), and combined stimulants (10 mM glucose + 5 mM DTT + 1 mM sodium dithionite) (**e**). Symbols (•) represent experimentally measured cumulative H_2 production, and solid lines represent the fitted modified Gompertz model. Data points are presented as mean ± SD (n = 3). The fitted parameters A, R_m, and λ represent the maximum H_2 production potential (mL·L^{-1}), maximum H_2 production rate (mL·L^{-1}·h^{-1}), and lag phase (h), respectively.

The enhancement of H_2 production was accompanied by a marked reduction in O_2 accumulation and an increase in hydrogenase activity (Table 2). The combined treatment exhibited the lowest O_2 production rate of 1.612 ± 0.060 mL·L^{-1}·h^{-1} and maintained a low cumulative O_2 yield of 10.405 ± 0.480 mL·L^{-1} compared with the untreated control with 20.176 ± 0.850 mL·L^{-1}. Glucose alone also substantially reduced O_2 accumulation to 10.553 ± 0.550 mL·L^{-1}, whereas sodium dithionite and DTT resulted in intermediate O_2 levels of 15.716 ± 0.750 and 16.139 ± 0.800 mL·L^{-1}, respectively. Consistent with the H_2 production results, the combined treatment exhibited the highest hydrogenase activity of 0.437 ± 0.041 mL·L^{-1}·min^{-1}, representing approximately a 168% increase over the untreated control of 0.163 ± 0.015 mL·L^{-1}·min^{-1}.

Table 2. Effects of selected single and combined stimulants on H_2, O_2 production and hydrogenase activity of *A. halophytica* incubated in $BG11_0$ medium supplemented with Turks Island salt solution under dark anaerobic conditions. Values are presented as mean ± SD (n = 3). Different superscript letters within the same column indicate significant differences according to Duncan's multiple range test at $p < 0.05$.

Treatment	Maximum H_2 Production Rate (mL·L^{-1}·h^{-1})	Maximum O_2 Production Rate (mL·L^{-1}·h^{-1})	Maximum Cumulative H_2 Yield (mL·L^{-1})	Maximum Cumulative O_2 Yield (mL·L^{-1})	Hydrogenase Activity (mL·L^{-1}·min^{-1})
No treatment (control)	0.218 ± 0.012 ^{e}	4.214 ± 0.120 ^{e}	3.043 ± 0.120 ^{e}	20.176 ± 0.850 ^{e}	0.163 ± 0.015 ^{c}
10 mM glucose	0.371 ± 0.018 ^{b}	1.729 ± 0.065 ^{b}	6.394 ± 0.502 ^{b}	10.553 ± 0.550 ^{b}	0.276 ± 0.024 ^{b}
1 mM sodium dithionite	0.311 ± 0.016 ^{c}	2.617 ± 0.060 ^{c}	4.528 ± 0.380 ^{c}	15.716 ± 0.750 ^{c}	0.230 ± 0.021 ^{b}
5 mM dithiothreitol	0.307 ± 0.015 ^{d}	2.646 ± 0.062 ^{d}	4.502 ± 0.360 ^{d}	16.139 ± 0.800 ^{d}	0.228 ± 0.020 ^{b}
10 mM glucose + 5 mM DTT + 1 mM sodium dithionite	0.588 ± 0.028 ^{a}	1.612 ± 0.060 ^{a}	8.445 ± 0.750 ^{a}	10.405 ± 0.480 ^{a}	0.437 ± 0.041 ^{a}

4. Discussion

4.1. Effects of Different Stimulant Groups on H_2 Production

The screening experiment demonstrated that the effectiveness of exogenous stimulants in enhancing H_2 production by *A. halophytica* was strongly dependent on their biochemical function. Among the five stimulant groups evaluated, glucose, sodium dithionite, and DTT significantly enhanced H_2 production, whereas phytohormones, vitamins, and mineral supplements provided no apparent benefit and, in several cases, markedly inhibited H_2 evolution. This pattern suggests that H_2 production in *A. halophytica* under dark, nitrogen-deprived conditions is influenced primarily by carbon availability and intracellular redox conditions rather than by compounds that primarily regulate growth or serve as micronutrients. Thus, the stimulants that were most effective in the present study appear to act directly or indirectly on the metabolic and redox environment required for H_2 evolution.

Although the experiments were performed under dark, argon-purged conditions to minimize photosynthetic O_2 evolution, measurable O_2 accumulation was still detected in the vial headspace (Table 1). This observation should be interpreted cautiously and does not necessarily ensure that the cultures remained completely anaerobic throughout the experimental period. Because gas samples were collected from the vial headspace, the detected O_2 may have resulted from residual O_2 remaining after argon purging and/or O_2 generated or released during incubation. One possible biological source is intracellular reactive oxygen species (ROS) metabolism. In cyanobacteria, H_2O_2 can be decomposed by catalase into H_2O and O_2, providing a potential non-photosynthetic source of O_2 under dark conditions [22]. However, because neither H_2O_2 concentration nor catalase activity was measured in this study, this mechanism remains speculative and cannot be established as the direct source of the observed O_2. In addition, dissolved O_2 was not independently quantified; therefore, the measured values represent the net O_2 accumulated in the headspace rather than the total O_2 content of the culture system. The lower O_2 accumulation observed following

supplementation with glucose, sodium dithionite, and DTT may reflect mitigated net O_2 levels under the combined treatment. However, the specific contributions of residual O_2, biological O_2 generation, and gas–liquid partitioning cannot be distinguished based on the present experimental design. Therefore, the observed reduction should be interpreted as decreased headspace O_2 accumulation rather than direct evidence of enhanced O_2 scavenging. Nevertheless, this reduction is physiologically relevant because hydrogenases are highly sensitive to O_2, and even limited O_2 accumulation can compromise their catalytic activity [7]. The contrasting trends in H_2 and O_2 accumulation across the treatments therefore suggest that maintaining a sufficiently reducing and low-O_2 environment may be an important prerequisite for efficient H_2 evolution. Future experiments incorporating abiotic blanks, killed-cell controls, leakage tests, and simultaneous measurements of dissolved and headspace O_2 would help clarify the origin and dynamics of O_2 under these experimental conditions.

The stimulatory effect of glucose is consistent with previous reports showing that exogenous carbon sources can enhance H_2 production by increasing substrate availability and generating reducing equivalents through glycolytic and fermentative pathways [13]. In contrast, the limited or negative effects of phytohormones may reflect the physiological state of the cells. Compounds such as cytokinins, gibberellins, and indole-3-acetic acid (IAA) are primarily associated with regulation of growth, photosynthetic activity, and cellular stress responses in cyanobacteria and microalgae [23,24]. Under nitrogen-starved, dark, and anaerobic conditions, however, cellular priorities shift from growth toward survival and energy conservation. Consequently, stimulation of growth-related regulatory pathways may not translate into increased H_2 production and could instead divert cellular resources away from H_2-generating metabolism. Similarly, the vitamin and mineral supplements tested did not improve H_2 production and, in several supplements, were accompanied by increased O_2 accumulation. Although vitamins and minerals are essential for cellular metabolism, their addition does not necessarily enhance H_2 production when the major limitation lies in electron availability or redox regulation. In some cases, excessive supplementation may disturb cellular homeostasis or promote reactions that increase intracellular oxidative pressure. The observed responses therefore emphasize that increasing the availability of individual nutrients is insufficient to improve H_2 production unless the overall metabolic and redox conditions remain favorable for hydrogenase activity.

4.2. Enhancement of H_2 Production by Glucose Supplementation

Glucose supplementation substantially enhanced H_2 production by *A. halophytica*, with 10 mM glucose yielding the highest H_2 yield among the concentrations tested. This response indicates that glucose can function as an effective external carbon and electron source under dark, nitrogen-deprived conditions. During nitrogen starvation, glycogen degradation provides endogenous carbon and reducing power, while exogenous glucose can supplement this carbon supply and increase flux through glycolytic and fermentative pathways [9,17]. Enhanced glycolytic activity would be expected to increase the formation of pyruvate and reducing equivalents, including NADH, which can subsequently support electron transfer toward H_2-producing reactions [17]. The increased H_2 production observed following glucose supplementation is therefore consistent with a shift toward greater availability of carbon-derived reducing power.

The simultaneous decrease in O_2 production at 10 mM glucose provides additional evidence that the treatment created a more favorable environment for H_2 evolution. Because hydrogenases are strongly inhibited by O_2, limiting O_2 accumulation can increase the fraction of enzyme activity directed toward H_2 production [7]. The improvement in H_2 production may therefore have resulted from the combined effects of enhanced elec-

tron availability and reduced O_2-associated inhibition rather than from increased carbon availability alone. However, the beneficial effect of glucose was not maintained at higher concentrations. Increasing the glucose concentration beyond 10 mM resulted in a pronounced decline in H_2 production, indicating that carbon availability was not the sole determinant of H_2 productivity. Excess glucose may disrupt metabolic homeostasis by increasing osmotic pressure and altering carbon partitioning toward competing metabolic pathways or extracellular fermentation products [9]. Such metabolic diversion could reduce the proportion of reducing equivalents available for H_2 production. Notably, the substantial increase in O_2 production at 20 and 50 mM glucose further suggests that excessive glucose disturbed the cellular redox balance or stimulated metabolic processes capable of contributing to O_2 accumulation. Thus, the response to glucose appears to be concentration-dependent, with 10 mM providing a favorable balance between increased carbon/electron supply and maintenance of a low-O_2, reducing environment. These findings highlight the importance of optimizing carbon availability rather than simply maximizing substrate concentration for improving dark anaerobic H_2 production by *A. halophytica*.

4.3. Roles of Sodium Dithionite and DTT in Improving H_2 Production

Among the reducing agents tested, sodium dithionite and DTT significantly enhanced H_2 production, indicating that modulation of the redox environment can favor H_2 evolution under dark, nitrogen-deprived conditions. Sodium dithionite acts as an O_2 scavenger that eliminates O_2, thereby initiating hydrogenase activity and enhancing H_2 production [15,16]. The reduction in O_2 accumulation observed at 1 mM sodium dithionite contributed to enhanced H_2 production by protecting O_2-sensitive hydrogenases from oxidative inactivation. Furthermore, the establishment of a more favorable redox environment may facilitate electron transfer processes associated with proton reduction. DTT exhibited a similar stimulatory effect but likely operated through a different mechanism. DTT is a widely used thiol-based reducing agent that preserves protein thiol groups in their reduced state, thereby helping maintain the activity of redox-sensitive enzymes under reducing conditions [25]. Hydrogenases contain iron–sulfur clusters that are essential for catalytic H_2 metabolism and electron transfer processes [7,26]. Therefore, the enhanced H_2 production observed following DTT supplementation may be associated with the maintenance of a more favorable reducing environment for H_2-producing enzymes, thereby supporting sustained H_2 evolution during prolonged incubation. Interestingly, excessive concentrations of both sodium dithionite and DTT reduced H_2 production. This observation indicates that an optimal redox balance is required for efficient H_2 evolution. Although reducing agents enhanced H_2 production at moderate concentrations, further increases resulted in lower H_2 yields. This finding suggests that excessively reducing conditions may disrupt cellular redox homeostasis and negatively affect metabolic processes involved in H_2 production. Maintaining an appropriate redox balance is important for efficient electron transfer and energy metabolism associated with H_2 evolution [17].

4.4. Enhancement of H_2 Production by Combined Stimulants

The modified Gompertz model provided a good representation of the observed H_2 accumulation profiles under all treatments (Figure 5). The estimated maximum H_2 production potential (A) ranged from 3.271 $mL \cdot L^{-1}$ in the control to 9.132 $mL \cdot L^{-1}$ in the combined treatment, while the maximum production rate (R_m) ranged from 0.064 to 0.181 $mL \cdot L^{-1} \cdot h^{-1}$. Notably, the experimentally measured cumulative H_2 production at 120 h corresponded to approximately 94.8–97.1% of the respective A values estimated by the model, indicating that H_2 accumulation had approached its asymptotic production potential by the end of the incubation period. Consistently, approximately 90.0–93.2% of the estimated production

potential had already been reached by 72 h, whereas only a relatively small increase in cumulative H_2 production was observed thereafter. These results support the interpretation that H_2 accumulation approached a plateau after approximately 72 h. The combined treatment exhibited the highest A and R_2 values, which were approximately 2.8-fold higher than those of the control, indicating that the combined application of glucose, sodium dithionite, and DTT enhanced both the overall H_2 production potential and the maximum production rate. The agreement between the experimentally measured accumulation profiles and the model-derived parameters further supports the effectiveness of the combined treatment in enhancing H_2 production.

The most significant finding of this study was the enhancement of H_2 production achieved through the combined application of glucose, sodium dithionite, and DTT. The combined treatment produced a cumulative H_2 yield of 8.445 ± 0.750 mL·L^{-1} (Table 2), which was approximately 178% greater than that of the untreated control and substantially higher than any individual stimulant treatment. This result indicates that the three stimulants targeted complementary physiological processes that collectively enhanced H_2 production. The enhancement observed under combined supplementation suggests that glucose, sodium dithionite, and DTT target complementary physiological processes. A hypothetical metabolic model explaining these interactions for enhanced H_2 production in *A. halophytica* under nitrogen-deprived dark anaerobic conditions is presented in Figure 6. Under nitrogen starvation, cellular metabolism may shift from growth-associated processes toward glycogen degradation, glycolysis, and fermentative metabolism, potentially increasing the generation of NADH and other reduced electron carriers. Supplementation with glucose may further increase carbon availability and glycolytic activity, potentially increasing the supply of reducing equivalents available for hydrogen-producing pathways [11]. The degradation of intracellular carbon reserves together with exogenous glucose metabolism may increase the generation of reducing equivalents through glycolysis and associated fermentative pathways, thereby supporting H_2 production under nitrogen-deprived conditions [13]. Exogenous glucose may stimulate not only glycolytic metabolism but also the oxidative pentose phosphate pathway, thereby increasing NADPH generation available for hydrogen-producing reactions [13]. Collectively, these metabolic processes are proposed to increase intracellular electron flux toward the bidirectional [NiFe]-hydrogenase, which could increase proton reduction and H_2 evolution. Simultaneously, sodium dithionite scavenges dissolved O_2 and promotes anaerobic conditions favorable for hydrogenase activity [15,16]. The resulting reduction in intracellular O_2 may minimize oxygen-mediated inactivation of hydrogenase and facilitate continuous electron transfer to the catalytic site. DTT contributes to the maintenance of cellular redox homeostasis by preserving protein thiol groups in a reduced state [25]. Maintenance of intracellular redox homeostasis may also preserve the activity of redox-sensitive proteins involved in hydrogenase-mediated electron transfer. The bidirectional [NiFe]-hydrogenase of *A. halophytica* consists of a diaphorase module (HoxEFU), which accepts electrons from NAD(P)H, and a hydrogenase module (HoxHY), where electrons are utilized for proton reduction to produce H_2. Therefore, reducing equivalent availability while simultaneously minimizing oxygen-mediated inhibition and maintaining intracellular redox balance is expected to enhance hydrogenase activity. This proposed hypothetical mechanism is consistent with the present results, in which the combined treatment exhibited the highest hydrogenase activity together with the greatest H_2 production and the lowest O_2 accumulation (Table 2).

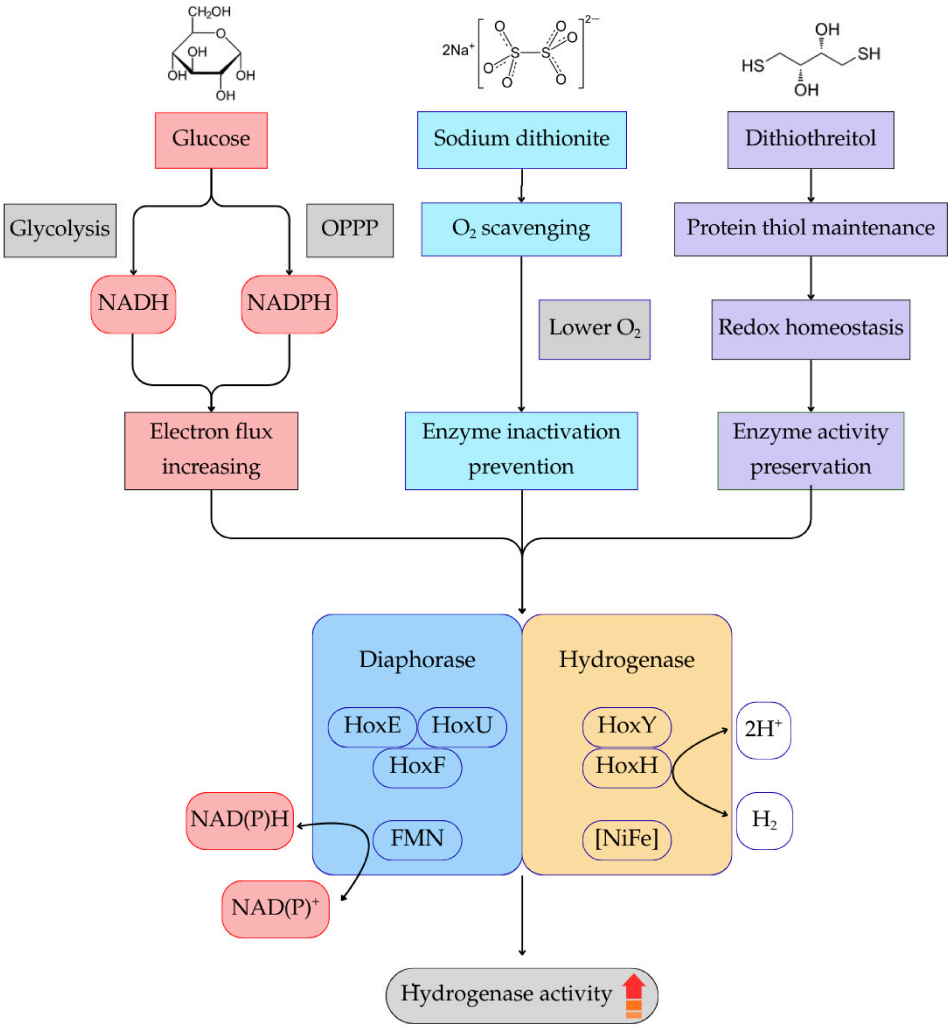


Figure 6. Proposed hypothetical mechanism illustrating the effects of glucose, sodium dithionite, and DTT on H_2 production in *A. halophytica* under nitrogen-deprived, dark and anaerobic conditions. Arrows indicate proposed relationships and potential metabolic or biochemical interactions leading to enhanced hydrogenase activity and H_2 production.

A possible molecular basis for the enhanced H_2 production may involve regulation of the bidirectional hydrogenase encoded by the *hox* operon. In *Aphanothece halophytica*, the *hoxE-hoxF-hoxU-hoxY-hoxH* genes are co-transcribed as a single operon, and nitrogen deprivation has been reported to increase *hoxH* transcript abundance concomitantly with enhanced H_2 production and hydrogenase activity [27]. Recently, nitrogen and potassium deprivation in *A. halophytica* were associated with increased hydrogenase activity and H_2 production, which was attributed, at least in part, to reduced O_2 availability under dark conditions [20]. Similar responses have been reported in other cyanobacteria. For example, low-O_2 conditions strongly induced *hox* transcription in *Anabaena*, while darkness further enhanced *hox* transcript accumulation under anaerobic conditions [28]. Likewise, the *hox* operon in *Synechocystis* sp. PCC 6803 was upregulated under low-O_2 conditions [29]. The sensitivity of hydrogenase to O_2 is an important factor determining its activity under H_2-producing conditions. Therefore, a decrease in local O_2 availability may reduce oxidative inhibition of the enzyme and help preserve its catalytically active state. In the present study, the use of sodium dithionite and DTT may have contributed to the maintenance of a more reducing environment, thereby creating conditions favorable for hydrogenase activity. Glucose may have further supported this process by providing an additional carbon and energy source and promoting the generation of reducing equivalents through

cellular metabolism. Thus, the enhanced H_2 production observed following combined treatment with glucose and reducing agents may reflect complementary effects involving increased availability of metabolic reducing equivalents and maintenance of a reducing environment favorable for hydrogenase activity. However, the present study did not directly determine *hox* transcription, intracellular O_2 concentration, or the redox state of hydrogenase. Therefore, it cannot be concluded that glucose, sodium dithionite, or DTT directly induced *hox* expression or directly protected hydrogenase at the molecular level. Rather, the observed enhancement is consistent with an indirect mechanism in which nitrogen deprivation and dark anaerobic conditions establish a low-O_2 environment, while the exogenous reducing agents further promote reducing conditions that may help preserve hydrogenase activity. Future studies combining *hox* gene-expression analysis, intracellular redox measurements, and direct assessment of hydrogenase protein or enzyme activity would be required to confirm this proposed mechanism.

To facilitate comparison with previous studies, the H_2 production rate and cumulative H_2 yield obtained in the present study were additionally expressed on a biomass-normalized basis (μmol H_2 g^{-1} dry weight h^{-1} and μmol H_2 g^{-1} dry weight, respectively; Table 3). The volumetric H_2 production data were converted to μmol H_2 based on the molar volume of H_2 under the corresponding experimental conditions and normalized to the dry biomass concentration. The H_2 production performance achieved in the present study was compared with previously reported enhancement strategies for *A. halophytica* (Table 3). The combined supplementation of glucose, sodium dithionite, and DTT resulted in a maximum H_2 production rate of 327.76 ± 15.61 μmol H_2 g^{-1} dry wt h^{-1}, which was approximately 2.4 to 8.3-fold higher than most previously reported strategies, including optimized seawater cultivation, photosystem II inhibition, combined nitrogen and potassium deprivation, cell immobilization, and bacterial co-cultivation [14,20,30]. Furthermore, the cumulative H_2 yield reached 4707.37 ± 418.06 μmol H_2 g^{-1} dry wt, representing the highest value among the conditions summarized in Table 3. The comparatively high H_2 production may be attributed to the integrated metabolic–redox strategy employed in this study. Unlike previous approaches that primarily targeted individual physiological or environmental constraints, the combined treatment simultaneously enhanced reducing-equivalent availability, reduced O_2 accumulation, and maintained a favorable redox environment for hydrogenase-mediated H_2 production. Although direct comparisons among studies should be interpreted cautiously because of differences in experimental conditions, these findings demonstrate the potential of integrated metabolic and redox regulation as an effective strategy for enhancing dark fermentative H_2 production in *A. halophytica*.

Table 3. Comparison of maximum H_2 production rates and cumulative H_2 yields of *Aphanothece halophytica* under different H_2-enhancement strategies (NA = Not Available).

Conditions	Maximum H_2 Production Rate	Cumulative H_2 Yield	References
Free cells			
$BG11_0$ with 10 mM glucose, 5 mM DTT, and 1 mM sodium dithionite, pH 7.4, 30 °C	0.588 ± 0.028 mL·L^{-1} or 327.76 ± 15.61 μmol H_2 g^{-1} dry wt h^{-1}	8.445 ± 0.750 mL·L^{-1} or 4707.37 ± 418.06 μmol H_2 g^{-1} dry wt	This study
$BG11_0$ with 25 μM simazine, pH 7.4, 30 °C	58.88 ± 0.22 μmol H_2 g^{-1} dry wt h^{-1}	356.21 ± 6.04 μmol H_2 g^{-1} dry wt	[30]
$BG11_0$-K, pH 7.4, 30 °C	40.87 ± 1.07 μmol H_2 g^{-1} dry wt h^{-1}	1261.96 ± 96.99 μmol H_2 g^{-1} dry wt	[20]
Co-cultivation			
Co-cultured with *Staphylococcus aureus* incubated in $BG11_0$ with 0.945 mmol C-atom L^{-1} glucose, 0.25 M NaCl, pH 7.4, 35 °C	132.49 ± 4.45 μmol H_2 g^{-1} dry wt h^{-1}	3248.51 ± 88.11 μmol H_2 g^{-1} dry wt	[14]

Although this strategy substantially enhanced H_2 production under the tested laboratory conditions, its practical and sustainability implications require careful consideration. Sodium dithionite and DTT are exogenous reducing agents, and their costs, stability under cultivation conditions, and potential environmental impacts were not evaluated in the present study. Glucose may represent a comparatively simpler alternative because it enhanced H_2 production without requiring an exogenous reducing agent; nevertheless, its economic and environmental advantages cannot be established from the present data. Furthermore, the feasibility of applying these compounds at larger scales, including their required dosages and potential effects on downstream processing, remains uncertain. Therefore, the present findings should be regarded as a laboratory-scale proof of concept rather than evidence of an immediately practical or sustainable H_2 production strategy. Further techno-economic and environmental assessments, together with scale-up studies and optimization of stimulant consumption, are needed to determine the commercial viability and sustainability of this approach.

5. Conclusions

Supplementing nitrogen-deprived, dark anaerobic cultures of the halotolerant cyanobacterium *A. halophytica* with selected reductants markedly enhanced H_2 production. Among five compound classes tested, only glucose, sodium dithionite, and DTT increased H_2 yields; phytohormones, vitamins, and minerals were ineffective. Optimal concentrations (10 mM glucose, 1 mM sodium dithionite, 5 mM DTT) maximized performance, and their combined application yielded a 178% increase in cumulative H_2 and the highest production rate relative to the untreated control. Enhancement coincided with reduced O_2 accumulation, consistent with improved operation of hydrogen-evolving enzymes. The synergy likely reflects concurrent optimization of electron supply (glucose), O_2 scavenging (dithionite), and intracellular redox balance (DTT). These results establish a simple, effective strategy to boost biohydrogen output and underscore the promise of halotolerant cyanobacteria as sustainable platforms for renewable H_2 production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/gases6030041/s1, Table S1: Analysis of variance of H_2 and O_2 production under different chemical supplement treatments presented in Table 1; Table S2: Statistical analysis of H_2 and O_2 production rates presented in Figure 1; Table S3: Statistical analysis of H_2 and O_2 production rates presented in Figure 2; Table S4: Statistical analysis of H_2 and O_2 production rates presented in Figure 3; Table S5; Statistical analysis of H_2 production rates presented in Figure 4; Table S6: Statistical analysis of H_2 and O_2 production, cumulative gas yields, and hydrogenase activity under different conditions presented in Table 2.

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Abbreviations

The following abbreviations are used in this manuscript:

DTT	Dithiothreitol
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
OPP	Oxidative pentose phosphate pathway

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