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# Dark fermentative hydrogen production and transcriptional analysis of genes involved in the unicellular halotolerant cyanobacterium *Aphanothece halophytica* under nitrogen and potassium deprivation

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The unicellular halotolerant cyanobacterium *Aphanothece halophytica* is known as a potential hydrogen (H<sub>2</sub>) producer. This study aimed to investigate the enhancement of H<sub>2</sub> production under nutrient deprivation. The results showed that nitrogen and potassium deprivation induced dark fermentative H<sub>2</sub> production by *A. halophytica*, while no differences in H<sub>2</sub> production were found under sulfur and phosphorus deprivation. In addition, deprivation of nitrogen and potassium resulted in the highest H<sub>2</sub> production in *A. halophytica* due to the stimulation of hydrogenase activity. The effect of adaptation time under nitrogen and potassium deprivation on H<sub>2</sub> production was investigated. The results showed that the highest H<sub>2</sub> accumulation of 1,261.96 ± 96.99 μmol H<sub>2</sub> g dry wt<sup>-1</sup> and maximum hydrogenase activity of 179.39 ± 8.18 μmol H<sub>2</sub> g dry wt<sup>-1</sup> min<sup>-1</sup> were obtained from *A. halophytica* cells adapted in the nitrogen- and potassium-deprived BG11 medium supplemented with Turk Island salt solution (BG11<sub>0</sub>-K) for 48 h. An increase in hydrogenase activity was attributed to the decreased O<sub>2</sub> concentration in the system, due to a reduction of photosynthetic O<sub>2</sub> evolution rate and a promotion of dark respiration rate. Moreover, nitrogen and potassium deprivation stimulated glycogen accumulation and decreased specific activity of pyruvate kinase. Transcriptional analysis of genes involved in H<sub>2</sub> metabolism using RNA-seq confirmed the above results. Several genes involved in glycogen biosynthesis (*glgA*, *glgB*, and *glgP*) were upregulated under both nitrogen and potassium deprivation, but genes regulating enzymes in the glycolytic pathway were downregulated, especially *pyk* encoding pyruvate kinase. Interestingly, genes involved in the oxidative pentose phosphate pathway (OPP) were upregulated. Thus, OPP became the favored pathway for glycogen catabolism and the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which resulted in an increase in H<sub>2</sub> production under dark anaerobic condition in both nitrogen- and potassium-deprived cells.

## KEYWORDS

*Aphanothece halophytica*, hydrogen production, gene expression, potassium deprivation, nitrogen deprivation

## 1 Introduction

Huge amounts of energy are used by humans in households, in industry, and in agriculture. Fossil fuel is the main energy source worldwide, but it is non-renewable and limited in supply. Due to annual increases in energy consumption, there is a high demand for fossil fuel. As a result, greenhouse gases produced from the combustion of fossil fuels have increased. This has led to environmental problems, including global warming. In addition, the amount of fossil fuel will not be sufficient for human energy consumption in the future. Hydrogen (H<sub>2</sub>) is an alternative energy carrier that can be used instead of the limited fossil fuel resources since its combustion releases a high heating value of 141.6 MJ kg<sup>-1</sup> (Perry 1963). This combustion of H<sub>2</sub> is an environmentally friendly process that does not produce greenhouse gases as by-products (Gupta and Parkhey 2017). H<sub>2</sub> can be produced by various types of microorganisms, such as purple bacteria, green sulfur bacteria, green algae, and cyanobacteria.

Cyanobacteria or blue-green algae are prokaryotic and photoautotrophic microorganisms that have oxygenic photosynthetic activity (Bothe et al., 2010). Cyanobacteria can convert abundant raw materials for photosynthesis such as water, sunlight, and CO<sub>2</sub> into carbohydrate and O<sub>2</sub>. Cyanobacteria can produce H<sub>2</sub> through many processes, depending on the cyanobacterial type and metabolism. Three enzymes are involved in H<sub>2</sub> metabolism in cyanobacteria, namely, bidirectional hydrogenase, uptake hydrogenase, and nitrogenase (Khetkorn et al., 2013). Despite the fact that cyanobacteria are capable of photobiological H<sub>2</sub> production, all enzymes involved are sensitive to O<sub>2</sub>. To sustain and increase the yield of H<sub>2</sub> production and prevent enzyme inactivity from O<sub>2</sub>, a two-stage H<sub>2</sub> production scheme was proposed (Ananyev et al., 2012). In the first stage, cyanobacteria are grown in complete media to accumulate biomass. Then, cells enter the second stage by incubating under stress conditions and produce H<sub>2</sub> when anaerobic conditions are established.

The deliberate depletion of nutrients in media is a technique used to induce H<sub>2</sub> production and is an effective way to maintain sustainable H<sub>2</sub> production in cyanobacteria (Srirangan et al., 2011). Under nitrogen deprivation, *A. halophytica* shows high H<sub>2</sub> production in dark anaerobic conditions (Taikhao et al., 2013; Taikhao et al., 2015). Moreover, nitrogen deprivation also induces H<sub>2</sub> evolution in *Oscillatoria brevis*, *Calothrix membranacea* (Lambert and Smith 1977), and *Anabaena siamensis* TISIR 8012 (Khetkorn et al., 2010; Taikhao and Phunpruch 2017). Under nitrogen deprivation, the accumulation of glycogen is carried out *via* photoautotrophic processes. The accumulated glycogen is degraded into glucose-6-phosphate with the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), the electron donor of [NiFe]-H<sub>2</sub>ase, and hydrogen is generated thereafter under dark anaerobic conditions (Ananyev et al., 2008). Previous research showed that under nitrogen deprivation, the bidirectional H<sub>2</sub>ase activity of *A. halophytica* was obviously induced during dark anaerobic incubation and the expression of *narB* encoding ferredoxin-nitrate reductase was downregulated (Phunpruch et al., 2016). Consequently, reduced nicotinamide adenine dinucleotide (NADH) was enhanced and subsequently H<sub>2</sub> was increasingly generated (Phunpruch et al., 2016). In addition, sulfur deprivation enhanced H<sub>2</sub> production in

*Gloeocapsa alpicola* (Antal and Lindblad 2005) and *Microcystis aeruginosa* (Rashid et al., 2009). Potassium deficiency increased H<sub>2</sub> production by *Synechocystis* sp. PCC 6803 (Ueda et al., 2016).

The unicellular halotolerant cyanobacterium *Aphanothece halophytica*, originally isolated from Solar Lake (Israel), has the ability to grow at high salinity at concentrations of NaCl up to 3 M (Takabe et al., 1988). Since it is an obligately halophilic strain that is unable to grow under NaCl-deprived conditions, it is a model microorganism for studying the halotolerance mechanism in cyanobacteria. *A. halophytica* is well able to produce dark fermentative H<sub>2</sub> under nitrogen depletion in enriched medium (Taikhao et al., 2013). It has advantages of growing and producing H<sub>2</sub> when cells were incubated in natural seawater without any supplementation of NaNO<sub>3</sub> (Taikhao et al., 2015). The bidirectional [NiFe]-H<sub>2</sub>ase was identified in *A. halophytica* (Phunpruch et al., 2016). Moreover, H<sub>2</sub> production by *A. halophytica* was enhanced by cell immobilization using alginate and agar (Pansook et al., 2016; Pansook et al., 2019a). In addition, *A. halophytica* treated with photosystem II inhibitors clearly increased H<sub>2</sub> production (Pansook et al., 2019b). Recently, simazine, an herbicide of the triazine class, was shown to significantly enhance H<sub>2</sub> production by *A. halophytica* under dark anaerobic condition (Pansook et al., 2022). In addition, sodium sulfide was an effective reducing agent for enhancing H<sub>2</sub> production by *A. halophytica* due to the reduced O<sub>2</sub> concentration in the system, thus increasing hydrogenase activity (Chinchusak et al., 2022).

In this study, we focused on H<sub>2</sub> production by *A. halophytica* under nutrient-deprived conditions and investigated H<sub>2</sub> metabolism under these conditions. Photosynthesis and carbon and nitrogen assimilation pathways in N- and K-deprived cells were studied by RNA-seq based transcriptome analysis. H<sub>2</sub>ase activity, glycogen content, and pyruvate kinase activity were also determined. The patterns of the transcriptome and experimental evidence were mutually supportive.

## 2 Materials and methods

### 2.1 Cyanobacterial cultivation

*Aphanothece halophytica* was grown photoautotrophically in a 250-ml Erlenmeyer flask containing 100 ml of BG11 medium (pH 7.4) (Rippka et al., 1979) supplemented with Turk Island salt solution (Garlick et al., 1977). The BG11 medium consisted of 17.6 mM NaNO<sub>3</sub> as a nitrogen source. The Turk Island salt solution contained high concentrations of minerals, including 0.5 M NaCl, 49 mM MgCl<sub>2</sub>•6H<sub>2</sub>O, 30 mM MgSO<sub>4</sub>•7H<sub>2</sub>O, and 8.9 mM KCl as main components. Cells were initially adjusted to a density of OD<sub>730</sub> at approximately 0.1 and subsequently shaken at 120 rpm on a rotary shaker at 30°C under a white-light fluorescence intensity of 30 μmol photons m<sup>-2</sup> s<sup>-1</sup> with a light (18 h/day): dark (6 h/day) cycle for 7 days.

### 2.2 Cyanobacterial adaptation condition under nutrient deprivation

H<sub>2</sub> production by *A. halophytica* was measured as part of its two-stage cultivation. Firstly, *A. halophytica* was grown in BG11 with Turk Island salt solution for 7 days to accumulate biomass. The seven-day-

old cells were harvested by centrifugation at  $8,000 \times g$  at 4°C for 10 min before resuspension in various types of single nutrient-depleted media: nitrogen-deprived BG11 supplemented with Turk Island salt solution (BG11<sub>0</sub>), phosphorus-deprived BG11 supplemented with Turk Island salt solution (BG11-P), potassium-deprived BG11 supplemented with Turk Island salt solution (BG11-K), and sulfur-deprived BG11 supplemented with Turk Island salt solution (BG11-S). To remove nitrogen in BG11<sub>0</sub>, NaNO<sub>3</sub> was omitted from BG11. In BG11-P, NaH<sub>2</sub>PO<sub>4</sub> was omitted from BG11. To remove potassium from BG11-K, KCl was omitted from Turk Island salt solution. In addition, K<sub>2</sub>HPO<sub>4</sub> was also omitted from BG11 and replaced by the addition of Na<sub>2</sub>HPO<sub>4</sub>. In BG11-S, ZnSO<sub>4</sub>•7H<sub>2</sub>O and CuSO<sub>4</sub>•5H<sub>2</sub>O were omitted from BG11 and replaced by ZnCl<sub>2</sub> and CuCl<sub>2</sub>, respectively. In addition, MgSO<sub>4</sub>•7H<sub>2</sub>O was omitted from both BG11 and Turk Island salt solution and replaced by the addition of MgCl<sub>2</sub>. Thereafter, the cell suspensions were incubated for 24 h before H<sub>2</sub> was measured, under anaerobic conditions.

### 2.3 H<sub>2</sub> measurement

*Aphanothece halophytica* adapted in each single nutrient-deprived media for 24 h was harvested by centrifugation and resuspended in a fresh medium. Five ml of cell suspension was transferred to a 10-ml glass vial. The vial was sealed with a rubber stopper and the cyanobacterial cells were made to enter anaerobic conditions by purging with argon gas for 10 min. Each cell suspension was then incubated at 30°C under darkness for 2 h before H<sub>2</sub> measurement was undertaken. H<sub>2</sub> measurement was performed using a Gas Chromatograph (Hewlett-Packard HP5890A, Japan) with a molecular sieve 5 Å 60/80 mesh packed column and a thermal conductivity detector using argon as the carrier gas (Baebprasert et al., 2010). H<sub>2</sub> production was expressed as μmol H<sub>2</sub> evolved per milligram dry cell weight. All experiments were done in triplicate.

### 2.4 Hydrogenase activity measurement

Bidirectional hydrogenase activity was determined by measuring H<sub>2</sub> evolution in the presence of dithionite-reduced methyl viologen. The reaction mixture comprised 1 ml of cell suspension and 1 ml of 25 mM sodium phosphate buffer (pH 7.6) containing 10 mM methyl viologen dichloride hydrate (Roche, Germany) and 40 mM sodium dithionite (Sigma, Germany). The mixture was incubated under argon atmospheric conditions at 30°C under darkness for 30 min. H<sub>2</sub> from headspace was subsequently analyzed by a gas chromatograph.

### 2.5 Dry cell weight and glycogen content determination

For each dry cell weight determination, 5 ml of cyanobacterial cell suspension was filtered through a glass microfiber filter GF/C (Whatman, United Kingdom). Sterile distilled water was used to wash the cells collected on the microfiber filter. Then, the filter containing cells was dried in an oven at 70°C until weight was constant. The dry cell weight was calculated. For glycogen content determination, glycogen extraction and hydrolysis were performed following the previous studied procedure (Ernst et al., 1984). *A. halophytica* cells that had been adapted in various media were

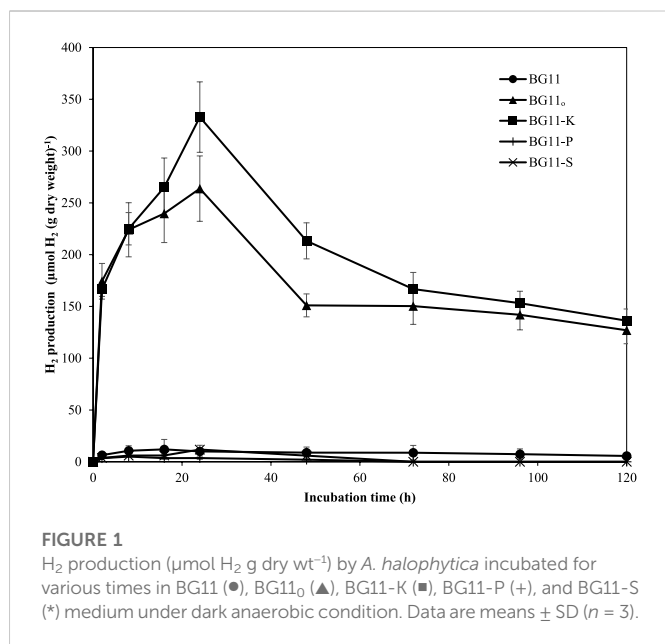
harvested by centrifugation at  $8,000 \times g$  at 4°C for 10 min and resuspended in fresh medium that contained chlorophyll *a* at a concentration of 300 μg chl *a* mL<sup>-1</sup>. Fifty μL of each cell suspension was added to 200 μL of 30% (w/v) KOH. The mixture was then incubated at 100°C in a water bath for 90 min. Cells were lysed by ultrasonication at 20% pulse for 5 min on ice. To precipitate glycogen, 600 μL of absolute ethanol was added into the extract. Each sample was kept on ice for 1 h. Glycogen was collected by centrifugation at  $12,000 \times g$  for 5 min at 4°C. Then, each glycogen pellet was washed twice by absolute ethanol and subsequently dried at 60°C for 10 min. Glycogen was resuspended in 300 μL of 100 mM acetate buffer (pH 4.75). For each sample, glycogen was digested into glucose by adding amyloglucosidase from *Aspergillus niger* (Sigma, United States) and α-amylase from *Aspergillus oryzae* (Sigma, Switzerland) at final concentrations of 4 and 8 units, respectively. The reaction was incubated at 25°C for 1 h. Insoluble membrane fragments were removed by centrifugation at  $12,000 \times g$  at 4°C for 5 min. Total sugar in each supernatant was determined by phenol-sulfuric acid assay (Dubois et al., 1956). Standard glycogen from bovine liver (Sigma, United States) (0–100 μg) was used. The glycogen content was calculated using a standard calibration curve. Each experiment was performed in triplicate.

### 2.6 Pyruvate kinase activity determination

Pyruvate kinase activity was determined indirectly by measuring the oxidation of NADH in the reaction catalyzed by lactate hydrogenase. The activity of pyruvate kinase was determined by a protocol previously described (Malcovati and Valentini, 1982). To prepare cell-free extract of *A. halophytica*, 100 ml of *A. halophytica* cell culture was harvested by centrifugation at  $8,000 \times g$  at 4°C for 10 min and subsequently resuspended in 1 ml of 10 mM (2-[4-(2-hydroxyethyl) piperazine-1-yl] ethanesulfonic acid (HEPES) buffer (pH 7.5). Cells were lysed using 20% pulse of ultrasonication on ice for 5 min. The cell-free extract was obtained after centrifugation at  $8,000 \times g$  at 4°C for 10 min. To assay pyruvate kinase activity, 50 μL of cell-free extract was added into 950 μL of reaction mixture containing 10 mM HEPES buffer, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 20 mM ADP (Adenosine 5'-diphosphate sodium salt) (Sigma, United States), 10 mM PEP (Phosphoenolpyruvic acid monopotassium salt) (Sigma, Switzerland), 5 mM NADH (β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate) (Sigma, United States), and 0.5 U of L-lactate dehydrogenase (Roche, Germany). The reaction mixture without PEP was used as a negative control. The decrease of NADH was measured spectrophotometrically at a wavelength of 340 nm at 25°C. One unit of pyruvate kinase activity is defined as the amount of enzyme that catalyzes the production of 1.0 μmol pyruvate from the substrate PEP in 1 min. The specific activity of pyruvate kinase was measured as activity per total protein concentration. Protein concentration was determined by Bradford assay (Bradford 1976).

### 2.7 mRNA sequencing by Illumina HiSeq/Novaseq or MGI2000

Seven-day old *A. halophytica* cells adapted in BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>K for 2 days were harvested by centrifugation at  $12,000 \times g$  at 4°C for 10 min. The total RNA of each sample was extracted using



QIAzol Lysis Reagent (Qiagen, United States). Then, total RNA was qualified and quantified using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, United States). Ribosomal RNA (rRNA) was removed by using a Ribo-Zero rRNA removal Kit (Epicentre, United States) following the manufacturer's instructions. The construction of the next generation sequencing library was conducted by Genewiz (China) according to the standard protocols. The different indices of libraries were multiplexed and loaded on an Illumina HiSeq/Novaseq instrument according to the instructions of the manufacturer (Illumina, United States). RNA sequencing was performed using the 2 × 150 paired end (PE) configuration. Image analysis and base calling were conducted using the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. All RNA sequencing and alignment processes were conducted by Genewiz (China).

## 2.8 Statistical analysis

All experiments were performed in triplicate. All data are shown as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used for analysis to the 95% confidence level with IBM SPSS version 19 software.

## 3 Results

### 3.1 H<sub>2</sub> production in deprived and limited media

The result showed that *A. halophytica* cells incubated in BG11<sub>0</sub> and BG11-K showed clearly higher H<sub>2</sub> production than those in BG11, BG11-P, and BG11-S (Figure 1). The highest H<sub>2</sub> accumulation of 332.82 ± 34.02 μmol H<sub>2</sub> g dry cell wt<sup>-1</sup> was shown by cells incubated in BG11-K for 24 h. It was 33-fold higher than that of cells cultivated in

normal BG11 medium (10.01 ± 5.92 μmol H<sub>2</sub> g dry cell wt<sup>-1</sup>) (Figure 1). In addition, cells incubated in BG11<sub>0</sub> accumulated maximum H<sub>2</sub> of 263.75 ± 31.58 μmol H<sub>2</sub> g dry cell wt<sup>-1</sup>, or 26-fold higher than that of cells incubated in normal BG11 medium (Figure 1). This study demonstrated the activation of H<sub>2</sub> production by *A. halophytica* under nitrogen and potassium deprivation.

In this study, the effects of various concentrations of nitrogen and potassium on H<sub>2</sub> production and H<sub>2</sub>ase activity by *A. halophytica* with focus on potassium and nitrogen deprivation was investigated. The results showed that during potassium deprivation, a decrease in nitrogen concentration resulted in higher H<sub>2</sub>ase activity and H<sub>2</sub> production by *A. halophytica* (Table 1). Similarly, during nitrogen deprivation, lower potassium concentration resulted in significantly higher H<sub>2</sub> production and H<sub>2</sub>ase activity by *A. halophytica* (Table 1). The highest H<sub>2</sub>ase activity with 120.05 ± 8.98 μmol H<sub>2</sub> g dry wt<sup>-1</sup> min<sup>-1</sup> and maximum H<sub>2</sub> accumulation with 507.51 ± 13.78 μmol H<sub>2</sub> g dry wt<sup>-1</sup> was shown in cells incubated under a combined K- and N-deprived conditions (Table 1). This H<sub>2</sub>ase activity and H<sub>2</sub> production was approximately 40 and 50-fold higher than those under normal conditions (incubated in BG11), respectively. The deprivation of both nitrogen and potassium together promoted H<sub>2</sub>ase activity, and thus resulted in higher H<sub>2</sub> production.

### 3.2 Time course of H<sub>2</sub>ase activity, H<sub>2</sub> production, and O<sub>2</sub> production under nitrogen and potassium deprivation

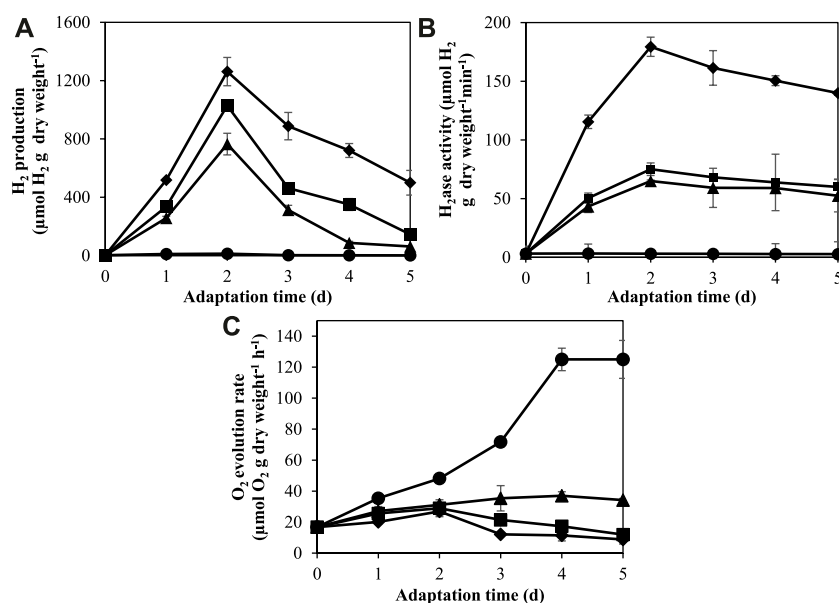
In this study, *A. halophytica* cells were adapted in BG11, BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K media under photoautotrophic conditions with light exposure (18 h/day) for 1, 2, 3, 4, and 5 days. The hydrogenase activity, H<sub>2</sub> production, and O<sub>2</sub> production of adapted cells were measured after incubation under dark anaerobic condition for 24 h. The result showed that under nitrogen and/or potassium deprivation, *A. halophytica* cells gave higher H<sub>2</sub> production and hydrogenase activity than cells under normal condition (Figures 2A, B). On the other hand, O<sub>2</sub> production was highest in cells adapted in normal BG11 medium (Figure 2C). The combined deprivation of nitrogen and potassium promoted higher H<sub>2</sub>ase activity and H<sub>2</sub> production in *A. halophytica* than did single nutrient deprivation. *A. halophytica* incubated under nitrogen and potassium deprivation showed maximum H<sub>2</sub> production rate of 40.87 ± 1.07 μmol H<sub>2</sub> g dry wt<sup>-1</sup> h<sup>-1</sup>. The highest H<sub>2</sub> production of 1,261.96 ± 96.99 μmol H<sub>2</sub> g dry wt<sup>-1</sup> was obtained in cells adapted in BG11<sub>0</sub>-K for 2 days (Figure 2A). The lowest O<sub>2</sub> evolution rate of 8.81 ± 2.93 nmol O<sub>2</sub> g dry wt<sup>-1</sup> h<sup>-1</sup> was obtained in cells adapted in BG11<sub>0</sub>-K after 3 days (Figure 2C). It was found that H<sub>2</sub> production was related to H<sub>2</sub>ase activity (Figures 2A, B). H<sub>2</sub>ase activity increased dramatically at the first 48 h of adaptation incubation and the highest H<sub>2</sub>ase activity with 179.39 ± 8.18 μmol H<sub>2</sub> g dry wt<sup>-1</sup> min<sup>-1</sup> was found in cells adapted in BG11<sub>0</sub>-K for 2 days (Figure 2B).

### 3.3 Glycogen accumulation under nitrogen and potassium deprivation

The glycogen content of *A. halophytica* cells adapted in BG11, BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K media for 1, 2, 3, 4, and 5 days was

**TABLE 1** H<sub>2</sub> production and H<sub>2</sub>ase activity by *A. halophytica* cells incubated in various types of media: BG11 supplemented with Turk Island salt solution (BG11), potassium-deprived BG11 supplemented with Turk Island salt solution (BG11-K) containing various concentrations of NaNO<sub>3</sub>, nitrogen-deprived BG11 supplemented with Turk Island salt solution (BG11<sub>0</sub>) containing various concentrations of KCl, and potassium- and nitrogen-deprived BG11 supplemented with Turk Island salt solution (BG11<sub>0</sub>-K) for 24 h. Data are means ± SD (n = 3). Different letters in columns indicate a significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at p < 0.05.

Type of media	KCl (mM)	NaNO <sub>3</sub> (mM)	H <sub>2</sub> production (μmol H <sub>2</sub> g dry weight <sup>-1</sup> )	H <sub>2</sub> ase activity (μmol H <sub>2</sub> g dry weight <sup>-1</sup> min <sup>-1</sup> )
BG11	8.93	17.6	10.55 ± 0.58 <sup>h</sup>	3.20 ± 0.22 <sup>f</sup>
BG11-K	0	17.6	332.58 ± 8.56 <sup>ef</sup>	51.38 ± 1.51 <sup>de</sup>
	0	13.2	354.44 ± 9.15 <sup>de</sup>	60.30 ± 4.68 <sup>cd</sup>
	0	8.8	379.38 ± 17.51 <sup>cd</sup>	62.97 ± 4.78 <sup>c</sup>
	0	4.4	449.32 ± 6.08 <sup>b</sup>	81.23 ± 7.03 <sup>b</sup>
BG11 <sub>0</sub>	8.93	0	260.34 ± 12.24 <sup>g</sup>	46.61 ± 3.30 <sup>e</sup>
	6.70	0	279.10 ± 17.43 <sup>g</sup>	51.83 ± 1.40 <sup>de</sup>
	4.47	0	313.99 ± 21.93 <sup>f</sup>	54.9 ± 3.10 <sup>cde</sup>
	2.23	0	388.94 ± 34.66 <sup>c</sup>	84.70 ± 6.65 <sup>b</sup>
BG11 <sub>0</sub> -K	0	0	507.51 ± 13.78 <sup>a</sup>	120.05 ± 8.98 <sup>a</sup>

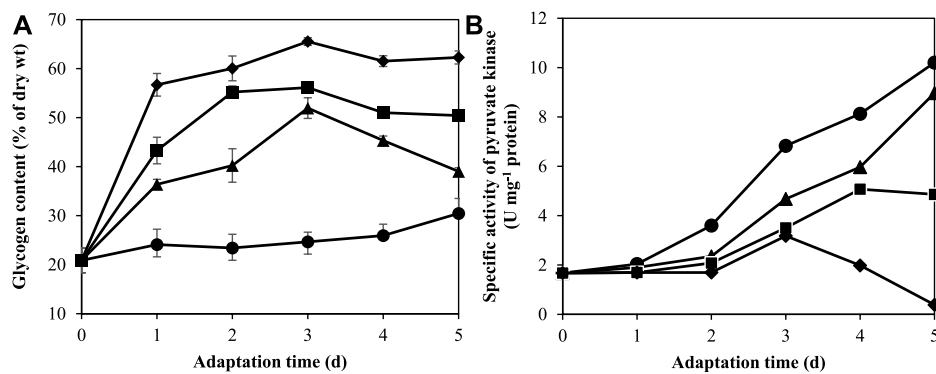


**FIGURE 2**

Effect of adaptation time on H<sub>2</sub> production (A), H<sub>2</sub>ase activity (B), and O<sub>2</sub> evolution rate (C) by *A. halophytica* cells. *A. halophytica* was adapted in various kinds of media for various times, BG11 (●), BG11<sub>0</sub> (▲), BG11-K (■), and BG11<sub>0</sub>-K (□) for 1–5 days before harvesting and resuspending in a fresh medium. H<sub>2</sub> production was measured in incubated cells under dark anaerobic condition for 24 h. Data are means ± SD (n = 3).

measured. The results revealed that under nitrogen and/or potassium deprivation, *A. halophytica* clearly produced and accumulated higher intracellular glycogen than under normal conditions. The glycogen content of cells adapted in BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K clearly increased during the first 3 days of adaptation, whereas in the case of BG11 adapted cells, it was almost constant over the period of adaptation (Figure 3A). The highest glycogen content at 65.53 ± 0.78% of cell dry wt was obtained in cells adapted in BG11<sub>0</sub>-K for 3 days (Figure 3A). It was

approximately three times higher than glycogen content of cells adapted in BG11 (24.66 ± 1.97% of cell dry wt) (Figure 3A). In the case of only single-nutrient deprivation, glycogen accumulated under potassium deprivation was higher than that under nitrogen deprivation. Under K-deprivation, cells accumulated the maximum glycogen content of 56.13 ± 0.66% of cell dry wt whereas under N-deprivation, cells accumulated the maximum glycogen content at 51.95 ± 2.11% of cell dry wt after 3 days of adaptation.



**FIGURE 3**

Effect of adaptation time on glycogen content (A) and specific activity of pyruvate kinase (B) by *A. halophytica* cells. *A. halophytica* was adapted in various kinds of media for various times, BG11 (●), BG11<sub>0</sub> (▲), BG11-K (■), and BG11<sub>0</sub>-K (□) for 1–5 days before harvesting and resuspending in a fresh medium. H<sub>2</sub> production was measured in incubated cells under dark anaerobic condition for 24 h. Data are means ± SD (*n* = 3).

### 3.4 Specific activity of pyruvate kinase under nitrogen and potassium deprivation

Pyruvate kinase (PK) is known to be activated by K<sup>+</sup> (Oria-Hernández et al., 2005). It has an important role in regulating the glycolytic pathway in carbohydrate metabolism. The measurement of pyruvate kinase (PK) activity was performed in *A. halophytica* cells adapted in BG11, BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K for 1, 2, 3, 4, and 5 days. The results showed that specific activity of PK was highest with 10.20 ± 0.60 U mg<sup>-1</sup> protein in *A. halophytica* adapted in BG11 for 5 days (Figure 3B). The deprivation of either nitrogen or potassium reduced specific activity of PK significantly compared with normal conditions (Figure 3B). Similarly, combined deprivation of both nitrogen and potassium had significant effects on PK activity. It was especially notable that after 5 days of adaptation time, cells clearly showed very low PK specific activity at 0.39 ± 0.01 U mg<sup>-1</sup> protein (Figure 3B).

### 3.5 Photosynthetic oxygen evolution rate and respiration rate under nitrogen and potassium deprivation

Since O<sub>2</sub> is a strong inhibitor of H<sub>2</sub>ase, the presence of O<sub>2</sub> in systems plays an important role in H<sub>2</sub> production. In cyanobacteria, O<sub>2</sub> is generated *via* oxygenic photosynthesis and the produced O<sub>2</sub> is consumed in cellular respiration *via* the electron transport chain. To investigate how O<sub>2</sub> evolution decreased under nitrogen and potassium deprivation, the photosynthetic O<sub>2</sub> evolution rate and respiration rate of *A. halophytica* cells adapted in BG11, BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K for 2 days was measured. The results showed that the highest photosynthetic O<sub>2</sub> evolution rate of 16.94 ± 0.64 nmol O<sub>2</sub> mg dry wt<sup>-1</sup> min<sup>-1</sup> was found in cells adapted in BG11. Under single or combined nitrogen and potassium deprivation, its photosynthetic O<sub>2</sub> evolution rate decreased (Table 2). The combined deprivation of both nitrogen and potassium resulted in the lowest photosynthetic O<sub>2</sub> evolution rate of 2.78 ± 0.09 nmol O<sub>2</sub> mg dry wt<sup>-1</sup> min<sup>-1</sup> (Table 2). On the contrary, O<sub>2</sub> consumption by respiration increased under nitrogen and/or potassium deprivation (Table 2). The ratio of photosynthetic O<sub>2</sub> evolution rate and respiratory rate was

calculated and evaluated. This ratio implies the quantity of O<sub>2</sub> in a vial container. The highest ratio between photosynthetic O<sub>2</sub> evolution rate and respiratory rate of 22.49 ± 0.81 was found in cells adapted in BG11 for 24 h whereas the lowest ratio of 2.57 ± 0.04 was shown in cells adapted in BG11<sub>0</sub>-K for 24 h (Table 2).

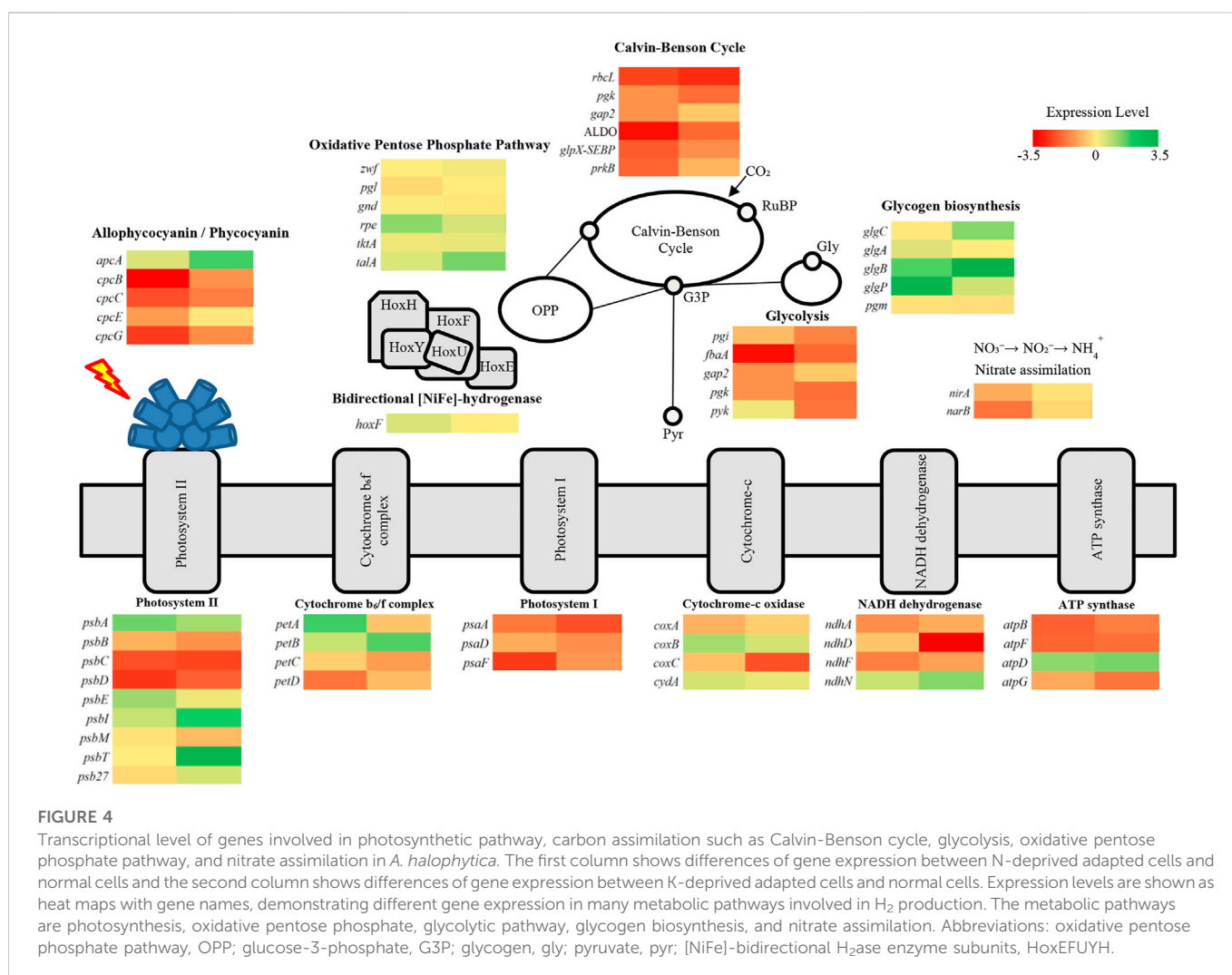
### 3.6 RNA-seq based transcriptome analysis of genes involved in photosynthetic, carbon, and nitrogen assimilation pathways under nitrogen and potassium deprivation

Gene expression based on the RNA-seq of *A. halophytica* under nitrogen and potassium deprivation was compared with that under normal conditions. The results are shown in Figure 4. Transcripts of genes involved in photosynthetic, carbon, and nitrogen assimilation pathways were analyzed. In this study, log<sub>2</sub>FoldChange (log<sub>2</sub>FC) was used to indicate the differences between transcript expression in the experimental and control groups. The results showed that genes encoding allophycocyanin and phycobilisome under N and K deprivation were expressed as log<sub>2</sub>FC ≤ -0.1. However, *ApcA*, the allophycocyanin alpha subunit encoded by *apcA*, was upregulated by 0.5 and 1.77 of log<sub>2</sub>FC under N and K deprivation, respectively. In addition, the expression of different genes involved in photosynthesis showed harmonic direction under N and K deprivation. Most of these genes were upregulated by log<sub>2</sub>FC ≤ 0.54. Especially, D1 protein encoded by *psbA* was upregulated by log<sub>2</sub>FC of 1.48 and 1.03 under N and K deprivation, respectively. However, the CP47, CP43 chlorophyll apoproteins, and P<sub>680</sub> reaction center D2 protein encoded by *psbB*, *psbC*, and *psbD* respectively were downregulated.

Under N and K deprivation, genes involved in the Calvin-Basham-Benson (CBB) cycle were downregulated (Figure 4). Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit encoded by *rbcL* was significantly down-regulated by log<sub>2</sub>FC of -2.46 and -1.83 under N and K deprivation, respectively. All genes in this pathway were differently expressed by log<sub>2</sub>FC ≤ -0.53. Likewise, several genes involved in glycolysis were downregulated under these starvation conditions. However, pyruvate kinase encoded by *pyk* was expressed differentially, as log<sub>2</sub>FC of 0.26 and -1.77 under N and K deprivation, respectively. Interestingly, most genes involved in

**TABLE 2** Photosynthetic O<sub>2</sub> evolution rate, dark respiration rate, and ratio of photosynthetic O<sub>2</sub> evolution rate and respiratory rate in *A. halophytica* cells incubated in BG11, BG11<sub>0</sub>, BG11-K, and BG11<sub>0</sub>-K under dark anaerobic condition for 24 h before measuring. Data are means ± SD (*n* = 3). Different letters in columns indicate a significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at *p* < 0.05.

Type of media	Photosynthetic O <sub>2</sub> evolution rate (nmol O <sub>2</sub> mg dry wt <sup>-1</sup> h <sup>-1</sup> )	Dark respiration rate (nmol O <sub>2</sub> mg dry wt <sup>-1</sup> h <sup>-1</sup> )	Ratio of photosynthetic O <sub>2</sub> evolution rate and dark respiration rate
BG11	16.94 ± 0.64 <sup>a</sup>	0.75 ± 0.03 <sup>c</sup>	22.49 ± 0.81 <sup>a</sup>
BG11 <sub>0</sub>	5.39 ± 0.87 <sup>b</sup>	1.12 ± 0.01 <sup>b</sup>	4.79 ± 0.73 <sup>b</sup>
BG11-K	5.47 ± 0.59 <sup>b</sup>	1.31 ± 0.04 <sup>a</sup>	4.17 ± 0.57 <sup>b</sup>
BG11 <sub>0</sub> -K	2.78 ± 0.09 <sup>c</sup>	1.08 ± 0.02 <sup>b</sup>	2.57 ± 0.04 <sup>c</sup>



glycogen biosynthesis were upregulated. For example, 1,4-alpha-glucan branching enzyme encoded by *glgB* was upregulated as log<sub>2</sub>FC of 1.69 and 4.37 under N and K deprivation, respectively. In the oxidative pentose phosphate pathway (OPP), some genes were upregulated under N and K deprivation. For example, *rpe* encoding ribulose phosphate 3-epimerase was upregulated by 1.23 and 0.52 of log<sub>2</sub>FC, respectively. In terms of nitrogen assimilation, as expected, ferredoxin-nitrite reductase encoded by *nirA* and *nirB* was downregulated as log<sub>2</sub>FC of -0.94 and -1.76, respectively, under

N-deprived conditions. On the contrary, no differences of expression of these genes were found under K-deprived conditions.

## 4 Discussion

Cyanobacteria are oxygenic microorganisms that can produce H<sub>2</sub> under dark anaerobic conditions, especially when they are incubated in nutrient-deprived media (Ananyev et al., 2008; Baebprasert et al.,

TABLE 3 Maximum dark fermentative H<sub>2</sub> production rate by *A. halophytica* incubated under various conditions.

Condition	Maximum H <sub>2</sub> production rate	References
Free cells incubated in BG11 <sub>0</sub> -K, dark, 120 rpm, pH 7.4, 30°C	40.87 ± 1.07 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	This study
Free cells incubated in BG11 <sub>0</sub> with 0.4 μM Fe <sup>3+</sup> , dark, 120 rpm, pH 7.4, 30°C	13.80 ± 0.373 μmol H <sub>2</sub> mg <sup>-1</sup> chl a h <sup>-1</sup>	Taikhao et al. (2013)
Free cells incubated in optimal seawater, dark, 120 rpm, pH 6.0, 35°C	82.79 ± 3.47 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	Taikhao et al. (2015)
Alginate-immobilized cells incubated in BG11 <sub>0</sub> , 120 rpm, pH 7.4, 30°C	0.532 μmol H <sub>2</sub> mg chl a <sup>-1</sup> h <sup>-1</sup>	Pansook et al. (2016)
Free cells incubated in BG11 <sub>0</sub> with 0.5 μM CCCP, dark, 120 rpm, pH 7.4, 30°C	39.50 ± 2.13 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	Pansook et al. (2019a)
Agar-immobilized cells incubated in BG11 <sub>0</sub> , dark, 120 rpm, pH 7.4, 40°C	135.54 ± 1.92 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	Pansook et al. (2019b)
Free cells incubated in BG11 <sub>0</sub> with 25 μM simazine, dark, 120 rpm, pH 7.4, 30°C	58.88 ± 0.22 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	Pansook et al. (2022)
Free cells incubated in BG11 <sub>0</sub> with 50 mM sodium sulfide, dark, 120 rpm, pH 7.4, 30°C	542.45 ± 35.99 μmol H <sub>2</sub> g dry wt <sup>-1</sup> h <sup>-1</sup>	Chinchusak et al. (2022)

2010; Min and Sherman 2010). In a previous study, the unicellular halotolerant cyanobacterium *A. halophytica* showed the ability to produce H<sub>2</sub> under nitrogen starvation (Taikhao et al., 2013; Taikhao et al., 2015; Phunpruch et al., 2016). Previously, H<sub>2</sub> production was shown to be catalyzed by bidirectional [NiFe]-H<sub>2</sub>ase (Phunpruch et al., 2016). The results in this study demonstrated that, apart from nitrogen starvation, potassium deprivation could also significantly enhance H<sub>2</sub> production by *A. halophytica* under dark anaerobic conditions compared with normal conditions (Figure 1). Potassium and nitrogen deprivation could induce glycogen catabolism with subsequent generation of increased electrons and hydrogenase activity was simultaneously induced after entering anaerobic conditions. After 24 h of incubation, H<sub>2</sub> production was decreased, as a result of the decreased number of electrons and the lower hydrogenase activity. Under potassium and nitrogen deprivation, biomass was not much changed due to the low level of nutrient concentration for cell division. However, *A. halophytica* incubated in seawater with the supplementation of 378 mmol C L<sup>-1</sup> glucose, 0.25 M NaCl, and 0.4 μM Fe<sup>3+</sup> at 35°C, pH six gave the highest H<sub>2</sub> production at day 8 of dark incubation under anoxic condition, and the high yield of H<sub>2</sub> was sustained at least up to 14 days (Taikhao et al., 2015).

Under potassium deprivations, a limitation of the NaNO<sub>3</sub> concentration available in BG11 medium induced both H<sub>2</sub>ase activity and H<sub>2</sub> production (Table 1). Similarly, under nitrogen deprivations, a limited KCl concentration in BG11 medium induced both H<sub>2</sub>ase activity and H<sub>2</sub> production (Table 1). In a previous study, it was shown that the presence of potassium reduced H<sub>2</sub>ase activity in *Synechocystis* 6,803, under dark anaerobic conditions, which might have been due to competitive utilization of limited NADPH for H<sub>2</sub> and organic acid production (Ueda et al., 2016). Moreover, it has been suggested that potassium deprivation decreased PSII activity, lowering the production of O<sub>2</sub>, which is an inhibitor of H<sub>2</sub>ase enzyme. Furthermore, potassium deprivation increased the degradation of starch in green alga *Scenedesmus obliquus* (Papazi et al., 2014) and in *Tetraspora* sp. CU2551 (Pewnuat et al., 2022). With nitrogen deprivation, H<sub>2</sub>ase activity increased in *Anabaena siamensis* TISTR8012, resulting in enhanced H<sub>2</sub> production (Taikhao and Phunpruch 2017). On the other hand, phosphorus and sulfur deprivation did not promote H<sub>2</sub> production by *A. halophytica*. In addition, potassium, sulfur, and phosphate deprivation was observed to likely have important effects on nucleic acids, protein synthesis, and other metabolic pathways in

cells, such as photosynthesis, cellular respiration, and H<sub>2</sub> metabolism (Warichanan and Phunpruch 2019). Moreover, a combination of nitrogen and sulfur depletion enhanced H<sub>2</sub> production in *Synechocystis* sp. strain PCC 6803 (Baebprasert et al., 2010) and in *Arthrospira* sp. PCC 8005 (Raksajit et al., 2012). Table 3 shows the comparison of maximum H<sub>2</sub> production rate by *A. halophytica* under various conditions. It was shown that deprivation of potassium together with nitrogen promoted higher H<sub>2</sub> production by *A. halophytica* than only nitrogen deprivation. However, H<sub>2</sub> production rate by free cells of *A. halophytica* in this study was lower than that of agar-immobilized cells (Pansook et al., 2019b) and free cells incubated in BG11<sub>0</sub> treated with 25 μM simazine (Pansook et al., 2022) and 50 mM sodium sulfide (Chinchusak et al., 2022).

H<sub>2</sub>ase, including the [NiFe]-H<sub>2</sub>ase enzyme in cyanobacteria, is usually sensitive to the presence of O<sub>2</sub> (McIntosh et al., 2011). The fundamental pathways involved in O<sub>2</sub> generation and consumption in cyanobacteria are photosynthesis and dark respiration, respectively. Our result showed that a combined deprivation of both nitrogen and potassium promoted H<sub>2</sub> production by reducing O<sub>2</sub> concentration in the vial (Figure 2). A decrease in O<sub>2</sub> concentration induced H<sub>2</sub>ase activity, resulting in a higher H<sub>2</sub> production (Figure 2). The balance of O<sub>2</sub> concentration in a system therefore plays a decisive role in H<sub>2</sub> production. In cyanobacteria, O<sub>2</sub> in the system is normally generated by photosynthetic activity of PSII. However, a measurement of H<sub>2</sub> production in this study was performed under darkness when photosynthetic activity should have been less than under light conditions. The deprivation of either nitrogen or potassium reduced the photosynthetic O<sub>2</sub> evolution rate by 3-fold, whereas combined deprivation of both nitrogen and potassium reduced the photosynthetic O<sub>2</sub> evolution rate by 6-fold (Table 2). A decrease in photosynthetic O<sub>2</sub> evolution rate caused the reduction of O<sub>2</sub> in the system. This in turn induced H<sub>2</sub>ase activity, resulting in higher H<sub>2</sub> production. In the case of nitrogen deprivation, the result was in agreement with a previous study showing the reduction of photosynthetic activities in *Synechococcus elongatus* PCC 7942 under nitrogen starvation (Choi et al., 2016). Potassium deprivation decreased photosynthetic pigments and activity in *Synechocystis* sp. strain PCC 6803 (Nanatani et al., 2015) and in *Anabaena torulosa* (Alahari and Apte 1998).

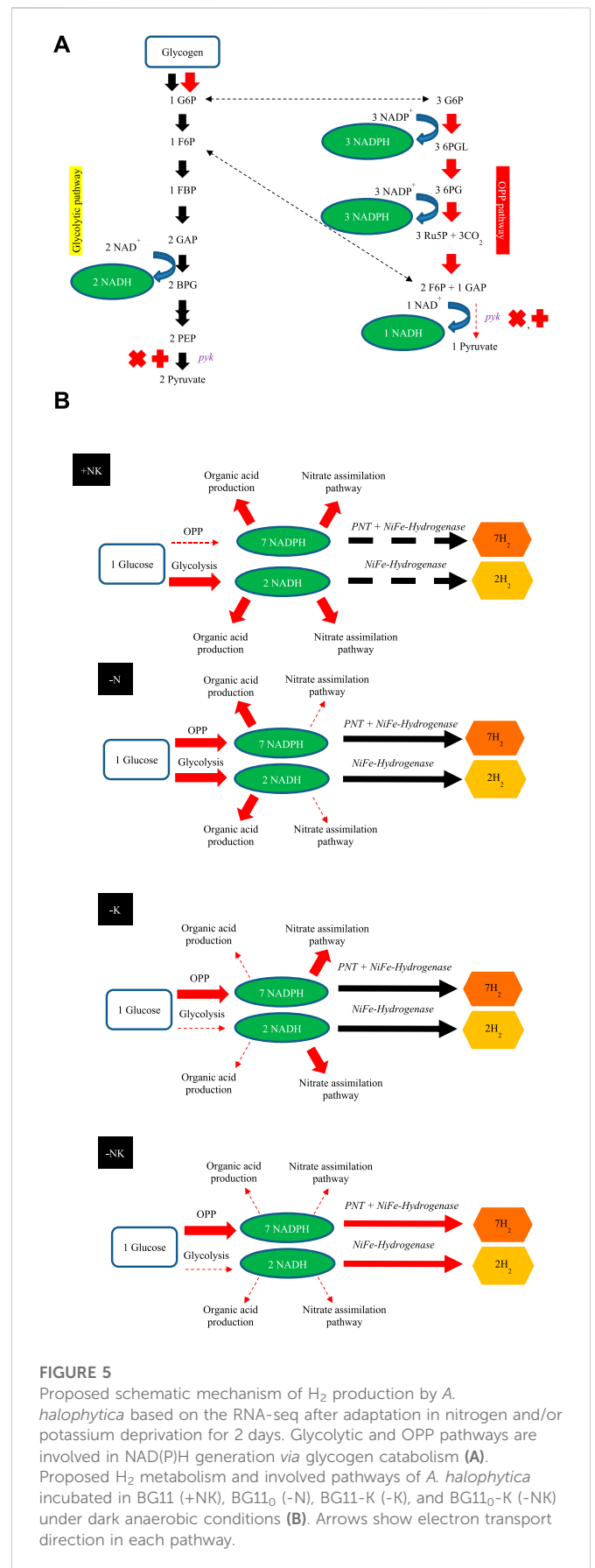
In addition, the produced O<sub>2</sub> can be consumed by dark intracellular respiration. An increased respiration rate for *A. halophytica* was observed in cells deprived of nitrogen or



potassium or both (Table 2). As a result, a decreased level of  $O_2$  in the vial containing cells incubated under nitrogen and potassium deprivation was obtained. The ratio of photosynthetic  $O_2$  evolution rate and dark respiration rate was used to monitor the level of  $O_2$  concentration in the system (Table 2). A higher ratio indicated higher activity of photosynthetic  $O_2$  evolution activity over respiration activity, suggesting the presence of high  $O_2$  concentration in the vial, and thus lower  $H_2$ ase activity and lower  $H_2$  production. A low ratio of photosynthetic  $O_2$  evolution rate to dark respiration rate was found in nitrogen- and potassium-free adapted cells (Table 2). This probably involved a combined effect of nitrogen and potassium deprivation suppressing related genes. In terms of photosynthetic  $O_2$  evolution: respiratory rate ratio (Table 2), the results implied that the lower the ratio, the lower the  $O_2$  concentration. Cells incubated in deprived media showed lower photosynthetic  $O_2$  evolution rate and dark respiration rate than did normal cells. Several genes encoding cytochrome *c* oxidase and NADH dehydrogenase were upregulated in cells under both nitrogen and potassium starvation (Figure 4). The results suggested that *A. halophytica* increased dark respiration under nitrogen and potassium starvation. However, no significant differences of expression of genes encoding respiratory electron transport system were reported in *Synechococcus elongatus* PCC 7942 under nitrogen deprivation (Choi et al., 2016).

$H_2$  production by *A. halophytica* was established by the two-stage regime: cyanobacterial growth followed by adaptation in nutrient deprivation condition to induce an increase of  $H_2$  production. Firstly, cells were grown in rich BG11 medium for 7 days to accumulate biomass. Then, the cells were made to enter the adaptation period, or the second stage, by incubation in nutrient-deprived media to accumulate glycogen under illumination and aerobic conditions. Subsequently, cells were transferred into a glass vial and  $H_2$  production was induced under dark anaerobic condition. The adaptation period during nutrient deficiency was very crucial since cells incubated in deprived media needed to accumulate a high content of glycogen, or other chemical compounds, in order to provide electrons for  $H_2$ ase to produce  $H_2$ . Individually and combined nitrogen and potassium deprivation were chosen for this study. The result showed that  $H_2$ ase activity and  $H_2$  production was highest in cells adapted in nitrogen- and potassium-deprived media for 48 h (Figures 2A, B), suggesting a high accumulation of glycogen in adapted cells. Glycogen in the adapted cells was extracted and determined. Glycogen was seen to have accumulated more in cells incubated in nitrogen- and potassium-deprived media compared to that of cells incubated in BG11 (Figure 3B). The highest glycogen was found in combined nitrogen- and potassium-free cells during the adaptation period (Figure 3B). This result was confirmed with transcriptional analysis. Transcriptional analysis by RNA-seq showed that the *glgA* and *glgB* genes encoding the starch synthase and 1,4-alpha-glucan branching enzymes, respectively, were upregulated under both nitrogen and potassium deprivation (Figure 4). The results suggested that a lack of nitrogen and/or potassium causes an increase in glycogen content, giving rise to higher  $H_2$  production (Taikhao et al., 2015).

Under dark anaerobic condition, cyanobacterial  $H_2$  can be produced via [NiFe]  $H_2$ ase reduced by NAD(P)H (Gutekunst



**FIGURE 5**

Proposed schematic mechanism of  $H_2$  production by *A. halophytica* based on the RNA-seq after adaptation in nitrogen and/or potassium deprivation for 2 days. Glycolytic and OPP pathways are involved in NAD(P)H generation via glycogen catabolism (A). Proposed  $H_2$  metabolism and involved pathways of *A. halophytica* incubated in BG11 (+NK), BG11<sub>0</sub> (-N), BG11-K (-K), and BG11<sub>0</sub>-K (-NK) under dark anaerobic conditions (B). Arrows show electron transport direction in each pathway.

et al., 2014) and reduced ferredoxin (Meuer et al., 1999; Gutekunst et al., 2014). The main sources of reductants for H<sub>2</sub> generation under dark anaerobic condition are NADH and reduced ferredoxin from glycolysis and NADPH from catabolism of accumulated glycogen, provided by oxidative pentose phosphate (OPP) pathway (Kumaraswamy et al., 2013). In this study, the specific activity of PK in glycolytic pathway decreased in *A. halophytica* under K deprivation (Figure 3A). The specific activity of PK in K- and NK- free adapted cells was less than that in abundant and N-free adapted cells throughout the period of adaptation time. PK is known to be induced by K<sup>+</sup> and it is less activated when K is absent in a system (Oria-Hernández et al., 2005). Therefore, this associated enzyme regulation might have controlled the flux of glycogen catabolism in *A. halophytica*. Furthermore, GAPDH-2 has a major role in the CBB cycle, using NADPH as a preferred electron donor to reduce 1,3-bis-phosphoglycerate to GAP (Koksharova et al., 1998). Under potassium deprivation, transcriptional analysis revealed that *pyk* and *gap2* encoding PK and GAPDH-2, respectively, were downregulated (Figure 4). Consequently, in potassium-free adapted cells, it could be suggested that upper-glycolytic metabolites could be in excess, especially glucose-6-phosphate (G6P). G6P is a broken-down molecule of glycogen, and the branching point between glycolysis and OPP pathway. For a reduced activity of both PK and GAPDH-2, it could be the case that G6P preferentially enters the OPP pathway, generating more NADPH and using less NADPH in CBB and glycolysis. Therefore, OPP pathway may well be the favored pathway for H<sub>2</sub> production in potassium-deprived adapted cells of *A. halophytica* under dark anaerobic conditions. In a previous study, the *pykF* knocking out *Escherichia coli* mutant provided higher activity of enzymes in OPP, but the activities of glycolytic enzymes decreased compared with those in wild-type cells (Siddiquee et al., 2004). Moreover, low activity of pyruvate kinase was shown to promote respiration in yeast (Grüning et al., 2011). Prior to this study, H<sub>2</sub> production was induced in nitrogen-free cells because the expression of genes involved in glycogen catabolism had increased (Osanai et al., 2006). Moreover, several genes in the OPP pathway were extremely upregulated in *Synechocystis* sp. PCC 6803 incubated in nitrogen-free medium (Osanai et al., 2006). This was probably due to G6P being degraded through the OPP pathway. It seems that glycogen catabolism might preferentially take place via the OPP pathway, producing more NAD(P)H compared to that via the glycolytic pathway in cells incubated in both nitrogen- and potassium-deprived conditions. Therefore, the described effects of both nitrogen and potassium deprivation on H<sub>2</sub> production may have been synergistic, and accelerated H<sub>2</sub> production in *A. halophytica* under dark anaerobic conditions.

The schematic H<sub>2</sub> production by *A. halophytica* during deprivation of individual nitrogen and potassium, and combined nitrogen and potassium, under dark anaerobic conditions is shown in Figure 5. The reductants involved in H<sub>2</sub> metabolism by cyanobacteria, NADH, and NADPH are generated mainly by the glycolytic and OPP pathways (Kumaraswamy et al., 2013). In the former, one molecule of G6P can provide two molecules of NADH whereas seven molecules of reduced pyridine nucleotides can be produced from the same molecule of G6P (6 NADPH per glucose and one NADH per glucose) via the OPP pathway (Kumaraswamy et al., 2013). One molecule of

NAD(P)H can be oxidized by H<sub>2</sub>ase to provide one molecule of H<sub>2</sub>. Therefore, the reduced pyridine nucleotides from the OPP pathway can produce more H<sub>2</sub> compared with that from the glycolytic pathway (seven molecules vs. two molecules of H<sub>2</sub>). In plentiful conditions or in the case of the BG11 (Figure 5B (+NK)), cells were more likely to generate organic acid compounds than potassium-deprived adapted cells were. This corresponded with a previous study of *Synechocystis* sp. PCC 6803 that suggested that, under dark anaerobic conditions, where NADPH was limited, there was a competing demand to consume NADPH for H<sub>2</sub> and organic acid production. In the presence of potassium, cells preferred to utilize NADPH for production of organic acids (Ueda et al., 2016). Therefore, NAD(P)H is normally used for organic acid production and the nitrate assimilation pathway rather than H<sub>2</sub> production. In nitrogen-deprived cells, it may be that glycogen catabolism occurs via both the OPP and glycolytic pathway, as shown in *Synechocystis* sp. PCC 6803 (Osanai et al., 2006). In potassium-free cells, PK activity (Figure 3) and the gene expression of *pyk* encoding PK was lower than that found in normal cells (Figure 4). Consequently, glycogen was mainly degraded via the OPP pathway and provided more NADPH for H<sub>2</sub> production.

## 5 Conclusion

Under dark anaerobic conditions, the maximum H<sub>2</sub> production of 1,261.96 ± 96.99 μmol H<sub>2</sub> g dry wt<sup>-1</sup> and the maximum hydrogenase activity of 179.39 ± 8.18 μmol H<sub>2</sub> g dry wt<sup>-1</sup> min<sup>-1</sup> was found in *A. halophytica* cells incubated in the nitrogen- and potassium-deprived BG11 medium supplemented with Turk Island salt solution for 48 h. The increased hydrogenase activity was due to a reduction of O<sub>2</sub> in the system, resulting from the lower photosynthetic O<sub>2</sub> evolution and higher dark respiration. Under nitrogen and potassium deprivation, *A. halophytica* cells promoted the production and accumulation of glycogen and reduced pyruvate kinase activity. Transcriptional analysis by RNA-seq helped to understand the effect of nitrogen and potassium deprivation on H<sub>2</sub> production by *A. halophytica*. Several genes involved in glycogen biosynthesis (*glgA*, *glgB*, and *glgP*) were upregulated in both nitrogen- and potassium-deprived cells. However, *pyk* and most genes that regulated enzymes in the glycolytic pathway were down-regulated in both nitrogen- and potassium-deprived cells. Interestingly, most genes that regulated enzymes in the oxidative pentose phosphate pathway (OPP) were upregulated. Accordingly, the OPP was suggested as a promising pathway for enhancing H<sub>2</sub> production under dark anaerobic conditions in both nitrogen- and potassium-deprived *A. halophytica* cells. This study indicated that combined nitrogen and potassium deprivation in media is a promising strategy to promote sustainable H<sub>2</sub> production by the halotolerant cyanobacterium *A. halophytica*.

## Data availability statement

The original contributions presented in the study are included in the article/SupplementaryMaterial; further inquiries can be directed to the corresponding author.

## Author contributions

SP contributed to conception and design of the study. SP received the research grant. NC performed the experiments and statistical analysis. NC wrote the first draft of the manuscript. SP and NC wrote sections of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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