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Enhanced dark fermentative H_2 production by agar-immobilized cyanobacterium *Aphanothece halophytica*

Sunisa Pansook[1] · Aran Incharoensakdi[2,3] · Saranya Phunpruch[1,4]

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Abstract

Cell immobilization is one of the techniques used to improve H_2 productivity in cyanobacteria. In this study, H_2 production by immobilized cells of unicellular halotolerant cyanobacterium *Aphanothece halophytica* was investigated and optimized. The results showed that immobilized cells of *A. halophytica* had higher H_2 production than free cells under nitrogen-deprived condition. Among various support material types used, agar-immobilized cells showed the highest H_2 production rate. Under nitrogen deprivation, the optimal conditions of cell immobilization for H_2 production were 3% (*w*/*v*) agar concentration, 0.2 mg dry cell weight per mL of gel solution, and 0.125 cm^3 of agar cube. The optimum pH of medium and incubation temperature for H_2 production by agar-immobilized cells were pH 7.4 and 40 °C, respectively. Using a large glass vial and headspace volume resulted in enhancement of H_2 production by agar-immobilized cells. Finally, H_2 production by agar-immobilized cells was analyzed for three consecutive cycles. H_2 production could be maintained at the highest level after two cycles when half of immobilized cells were replaced with fresh immobilized cells. These findings indicate that the enhanced H_2 production of the unicellular halotolerant cyanobacterium *A. halophytica* can be achieved by immobilization method, thus providing the possibility to improve H_2 production by cyanobacteria in the future.

Keywords H_2 production · Immobilization · Cyanobacteria · *Aphanothece halophytica*

Introduction

Hydrogen gas (H_2) is considered as a clean and efficient energy carrier that can be used instead of current fossil fuels. H_2 combustion provides a high heating value of 141.6 MJ kg^{-1} (Perry 1963) and generates mainly non-carbon-based products which do not affect the environmental pollution and the ozone layer (Kotay and Das 2008). H_2 is mainly produced by the steam reforming and thermochemical processes; however, these processes require raw materials from diminished fossil fuel reserves. Besides, H_2 can also be produced by biological processes from various kinds of microorganisms depending on the typical metabolic pathways. In cyanobacteria, H_2 is mostly produced by electrons obtained from the degradation of storage carbohydrates under darkness rather than electrons from a water oxidation of a direct photolysis (Tamagnini et al. 2007; Ananyev et al. 2008). In some N_2-fixing cyanobacteria, H_2 is produced as a by-product from N_2 fixation process (Reddy et al. 1996; Chen et al. 2008).

The unicellular halotolerant cyanobacterium *Aphanothece halophytica* is a model organism for studying salt tolerance mechanism in cyanobacteria because it could tolerate a high salinity up to 3 M NaCl (Takabe et al. 1988). *Aphanothece halophytica* has previously been shown as one of high potential H_2-producing cyanobacteria (Taikhao et al. 2013, 2015). It produces H_2 mainly by electrons obtained from a dark fermentation of storage glycogen under anaerobic condition (Taikhao et al. 2013). H_2 production of *A. halophytica* is catalyzed by bidirectional hydrogenase encoded by five structural genes, *hoxE*, *hoxF*, *hoxU*, *hoxY*, and *hoxH* (Phunpruch et al. 2016). H_2 production and

✉ Aran Incharoensakdi
aran.i@chula.ac.th

✉ Saranya Phunpruch
saranya.ph@kmitl.ac.th

[1] Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand

[2] Laboratory of Cyanobacterial Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[3] Academy of Science, Royal Society of Thailand, Bangkok 10300, Thailand

[4] Bioenergy Research Unit, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand

in vivo bidirectional hydrogenase activity of *A. halophytica* are significantly increased under nitrogen-deprived condition (Taikhao et al. 2013; Phunpruch et al. 2016). In addition, *A. halophytica* can grow in natural seawater supplemented with 1.76 mM $NaNO_3$, one-tenth of $NaNO_3$ concentration in BG11, and can provide long-term H_2 production when incubated in natural seawater (Taikhao et al. 2015).

The immobilization of cells on a suitable support material can be used to enhance H_2 production efficiency of microorganisms. Cell immobilization can protect enzyme activity and/or cells from an external hostile environment by separating cells from a liquid phase (Bickerstaff 1995). Moreover, immobilization can prevent cells from forming clumps which lead to a breakage of the filaments (Anjana and Kaushik 2014). Cell entrapment in porous gels has been shown as the most popular technique for whole cell immobilization (Freeman 1984). H_2 production by immobilized cells has been investigated in some strains of cyanobacteria. In the filamentous cyanobacterium *Calothrix* sp. 336/3, sodium alginate–immobilized cells showed an increase of H_2 production efficiency and a prolonged H_2 production over several cycles (Leino et al. 2009). Immobilized cells of cyanobacterium *Microcystis aeruginosa* with agar allowed the maintenance of stable H_2 production (Rashid et al. 2009). H_2 production by immobilized cells of the filamentous cyanobacterium *Lyngbya perelegans* with agar and alginate has been more sustained than that by free cells (Anjana and Kaushik 2014).

To enhance the efficiency of H_2 production in *A. halophytica*, we investigated H_2 production by cells immobilized with various support materials. H_2 production of immobilized cells was optimized under various physiological conditions such as concentration and size of support materials, cell concentration, nutrient and mineral concentrations, pH of medium, incubation temperature, size of container, and headspace volumes. Finally, H_2 production of immobilized cells for three cycles was also investigated.

Materials and methods

Cyanobacterial strain and growth condition

The halotolerant unicellular cyanobacterium *Aphanothece halophytica* was cultivated 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. 1997). The initial cell concentration was adjusted to OD_{730} of approximately 0.1. The cyanobacterial cells were cultivated in an incubator shaker at a speed of 120 rpm at 30 °C under the light intensity of 30 μmol photons m^{-2} s^{-1} for 7 days.

Cell immobilization with various support materials

Aphanothece halophytica cells grown in BG11 supplemented with Turk Island salt solution for 7 days were harvested by centrifugation at 8000×*g* at 4 °C for 10 min, washed twice and resuspended in $NaNO_3$-deprived BG11 medium ($BG11_0$) supplemented with Turk Island salt solution. The cyanobacterial cells were immobilized with three different types of support materials: agar (Difco, USA), agarose (Bio Whittaker Molecular Application, USA), and κ-carrageenan (Marcel Carrageenan, Philippines). The cell suspension was added to the autoclave-sterilized agar, agarose, and κ-carrageenan solutions in water bath at 50 °C and mixed by vortexing. A final cell concentration was adjusted to 1 mg dry cell weight per mL of gel solution whereas the final concentration of agar, agarose, and κ-carrageenan solutions was 1.5% (*w*/*v*) in $BG11_0$ supplemented with Turk Island salt solution. The mixture was cooled to solidify the gel before cutting into square cubes of 0.5 × 0.5 × 0.5 (w × l × h) cm^3.

Optimization of cell immobilization for H_2 production

Aphanothece halophytica cells were immobilized with the selected support material. In this study, concentrations of support material (1–3% (*w*/*v*)) and cyanobacterial cells (0.2–5 mg dry cell weight mL^{-1}) including volumetric sizes of cubes with immobilized cells (0.0156–1 cm^3) were optimized. The immobilized cells of *A. halophytica* were transferred into a 20-mL glass vial and incubated in $BG11_0$ supplemented with Turk Island salt solution for 24 h under the light before measuring H_2 production using a gas chromatograph.

Effect of nutrient and mineral concentrations on H_2 production rate by immobilized cells

Aphanothece halophytica cells immobilized with the selected support materials were soaked in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing various independent concentrations of nutrients and minerals, 0–176 mM $NaNO_3$, 0.25–1 M NaCl, 0–150 mM $MgSO_4 \cdot 7H_2O$, 0–400 μM Fe^{3+}, or 0–100 μM Ni^{2+}. The immobilized cells were transferred into a 20-mL glass vial containing 9 mL of indicated media and subsequently incubated at 30 °C under light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h. After incubation, immobilized cells were purged with argon gas to enter anaerobiosis and incubated in the dark for 24 h. H_2 production measurement of cells was determined from the headspace in vials. In addition, the effects of pH of medium (pH 6–10) and incubation temperature (20–45 °C) on H_2 production by immobilized cells were also investigated.

Effect of volumetric size of glass vial, immobilized cells, and headspace on H_2 production rate by immobilized cells

The immobilized cells of *A. halophytica* were transferred into two volumetric sizes of glass vial (20 and 120 mL) and incubated in $BG11_0$ supplemented with Turk Island salt solution for 24 h under the light before measuring H_2 production using a gas chromatograph. The effects of different volumetric sizes of immobilized cells and headspace on H_2 production rate were also investigated.

Cycle of H_2 production by immobilized cells

The immobilized cells and free cells of *A. halophytica* were transferred into a 120-mL glass vial and incubated in $BG11_0$ supplemented with Turk Island salt solution at 30 °C under light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h. Cells were purged with argon gas and incubated at 40 °C under darkness. H_2 production of cells was determined for three cycles (36 h for each cycle). After finishing H_2 production measurement in each cycle, the medium in glass vial was removed and replaced with the fresh medium. In some cases, different volumes (30, 50, and 100%) of immobilized cells after the first cycle were removed and replaced with the same respective volume of fresh immobilized cells for the analysis of H_2 production in the second and third cycles. For free cell experiments, the culture after each cycle was centrifuged to harvest the cells before suspension with the new fresh medium for the next cycle. Similarly, the different volumes (30, 50, and 100%) of free cell culture after the first cycle were removed and replaced with the same respective volume of fresh free cells from 7-day culture before analyzing H_2 production in the second and third cycles. Then, cells were purged with argon gas to remove O_2. H_2 production was analyzed for three cycles.

Measurement of H_2 production

A total of 6 cm^3 cubes containing immobilized cells with 6 mg dry cell weight (equivalent to 1 mg dry cell weight per mL of gel solution) were transferred into a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. Glass vials were sealed with a rubber stopper with an aluminum rim. The cubes with immobilized cells were incubated in $BG11_0$ supplemented with Turk Island salt solution at 30 °C under light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h. Then, O_2 in the glass vial was removed by purging argon gas for 10 min. Immobilized cells were then incubated under darkness at 30 °C for 24 h. H_2 evolution of immobilized cells was determined by analyzing the headspace gas phase using a gas chromatograph (Hewlett-Packard HP5890A, Japan) with a molecular sieve 5 Å 60/80 mesh packed column and thermal conductivity detector as previously described (Taikhao et al. 2013). H_2 production was expressed as μmol H_2 per g dry weight per hour. Each H_2 production determination was performed with triplicate measurements.

Dry cell weight determination

Dry cell weight of *A. halophytica* was measured by filtration of 10 mL cell suspension through a GF/C glass microfiber filter (47 mm diameter) (Whatman, UK). The filter containing cells was washed twice with distilled water and dried at 70 °C in an oven before weighing using a four-digit balance. This drying step was repeatedly performed until a constant weight was obtained. The dry cell weight was calculated from the difference between the weight of filter with and without cells.

Statistical data analysis

The values of H_2 production by immobilized cells were statistically compared using a one-way analysis of variance (ANOVA) with Duncan's multiple range test. Differences between treatment are considered significant at a level of 0.05 ($p < 0.05$). Data were analyzed using IBM SPSS statistics 23 (IBM Corp, USA).

Results

Effect of support material types on H_2 production by immobilized cells

H_2 production by *A. halophytica* immobilized cells with three support materials (agar, agarose, and κ-carrageenan) was investigated. The square cubes containing cells immobilized with agar, agarose, and κ-carrageenan were prepared and used for the study. H_2 production of immobilized cells and free cells incubated in $BG11_0$ under darkness was monitored up to 7 days as shown in Fig. 1. For *A. halophytica* cells immobilized with 1.5% (*w*/*v*) agar, maximum accumulated H_2 at 164.08 ± 4.76 μmol H_2 g^{-1} dry weight was observed after anaerobic dark incubation for 2 days, followed by cells immobilized with 1.5% (*w*/*v*) κ-carrageenan and 1.5% (*w*/*v*) agarose, respectively. The results indicated that agar had the highest potential as the support material for H_2 production by immobilized cells of *A. halophytica*. At day 2 of anaerobic dark incubation, H_2 production by agar-immobilized cells was approximately fivefold higher than that by free cells (Fig. 1). It should be noted that agar-immobilized cells had the highest rate of H_2 production at 6.16 ± 0.08 μmol H_2 g^{-1} dry weight h^{-1} after 1 day of anaerobic dark incubation. Agar-immobilized cells were used for optimization of H_2 production in further experiments.

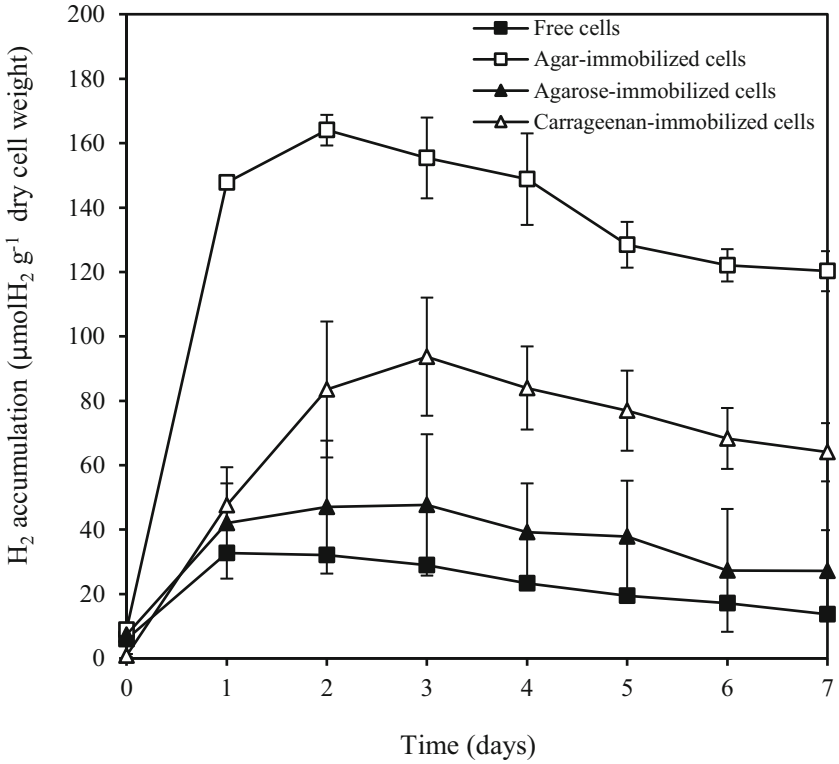

Fig. 1 Effect of support material types on H_2 accumulation by immobilized cells of *A. halophytica* under N deprivation. Harvested cells from 7-day culture suspended in N-deprived medium were immobilized with various support material types at 1.5% (*w*/*v*) concentration. The cell concentration was fixed at 1 mg dry cell weight per mL of gel solution. The cubes with immobilized cells were incubated at 30 °C in the light for 24 h before determination of H_2 production by further incubating the cubes for another 24 h in the dark. Free cell experiments were done in the same manner but without a procedure of cell immobilization

Effect of agar concentration on H_2 production by immobilized cells

Aphanothece halophytica cells were immobilized with either 1, 1.5, 2, 2.5, or 3% (*w*/*v*) agar concentration. The results showed that H_2 production of agar-immobilized cells was increased with an increase of agar concentrations. Cells immobilized with 3% (*w*/*v*) agar produced the highest H_2 production of 19.72 ± 0.76 μmol H_2 g^{-1} dry weight h^{-1} after anaerobic dark incubation for 1 day (Fig. 2a). Agar concentration lower than 1% (*w*/*v*) was difficult to handle due to its soft gel texture. On the other hand, some obstacles such as a lot of bubbles, high viscosity, and too rapid solidification were encountered when agar concentrations higher than 3% (*w*/*v*) were used. Thus, the final 3% (*w*/*v*) agar concentration was used for further experiments.

Effect of cell concentration on H_2 production by agar-immobilized cells

H_2 production rate by agar-immobilized cells with various final cell concentrations at 0.2, 0.5, 1, 2, 3, and 5 mg dry cell weight mL^{-1} was determined under dark anaerobic condition. The results showed that an increase in cell concentration resulted in a decrease of H_2 production (Fig. 2b). Agar-immobilized cells containing 0.2 mg dry cell weight mL^{-1} gave the highest H_2 production of 38.85 ± 1.91 μmol H_2 g^{-1} dry weight h^{-1} after anaerobic dark incubation for 1 day (Fig. 2b). Thus, the final cell concentration of 0.2 mg dry cell weight mL^{-1} was used for further experiments.

Effect of volumetric size of cubes with agar-immobilized cells on H_2 production

Agar gels cut into the square cubes with the same sizes of width, length, and height at 0.25, 0.50, 0.75, and 1 cm, corresponding to volumetric sizes of 0.0156, 0.125, 0.422, and 1 cm^3, respectively, were used for H_2 production experiments. The cubes of agar-immobilized cells with volumetric size of 0.125 cm^3 showed the highest H_2 production of 39.14 ± 1.51 μmol H_2 g^{-1} dry weight h^{-1} after anaerobic dark incubation for 1 day (Fig. 2c). This volumetric size was used for further experiments.

Effect of nutrient and mineral concentrations on H_2 production by immobilized cells

Aphanothece halophytica cells entrapped in 3% (*w*/*v*) agar gels with final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing various concentrations of $NaNO_3$, NaCl, $MgSO_4 \cdot 7H_2O$, Fe^{3+}, and Ni^{2+}. Under various $NaNO_3$ concentrations, agar-immobilized

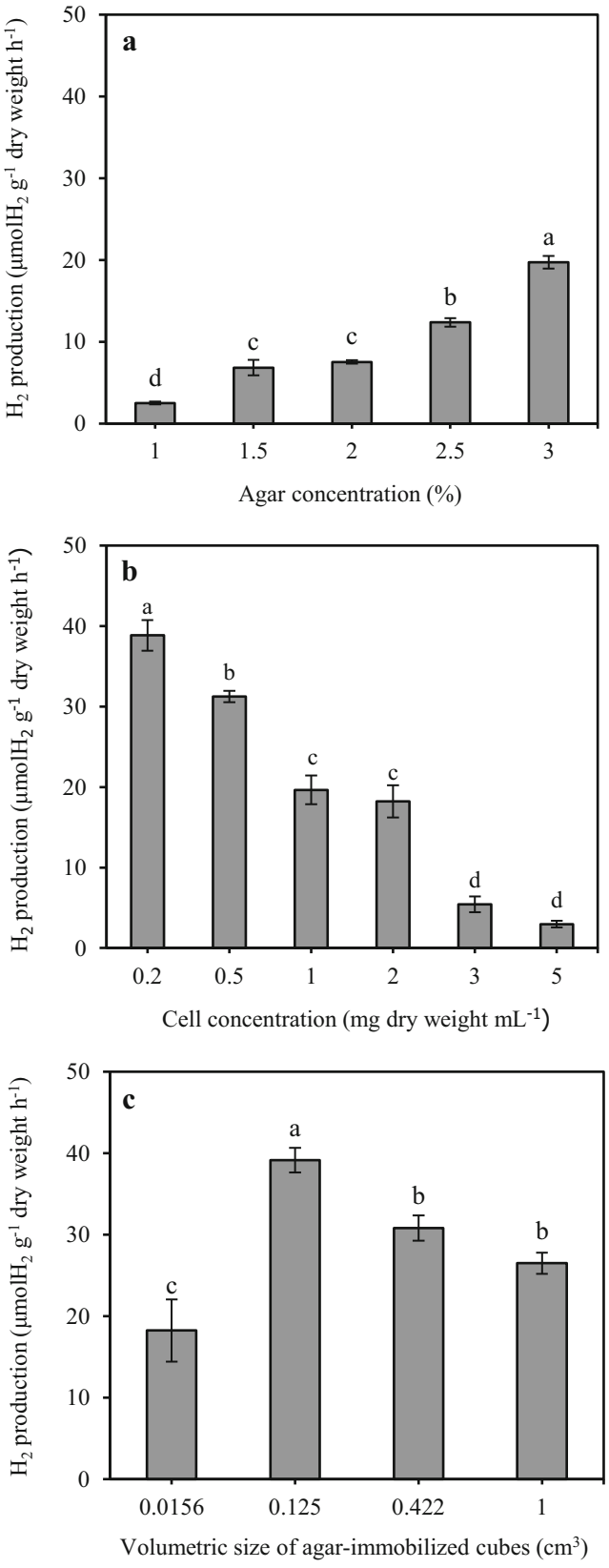

Fig. 2 Effect of agar concentration, cell concentration, and volumetric size of agar cubes with immobilized cells on H_2 production by *A. halophytica* under N deprivation. The agar cubes at various concentrations (**a**) with immobilized cells at 1 mg dry cell weight per mL gel solution were incubated at 30 °C in the light for 24 h before determination of H_2 production by further incubating the cubes for another 24 h in the dark. H_2 production was also measured in 3% (*w*/*v*) agar-immobilized cells with various cell concentrations (**b**) and various volumetric sizes of cubes with immobilized cells (**c**). Data are means ± SD ($n = 3$). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$

cells incubated in BG11 without $NaNO_3$ gave the highest H_2 production of 39.26 ± 0.38 μmol H_2 g^{-1} dry weight h^{-1} (Table 1). When $NaNO_3$ concentration was increased, H_2 production by agar-immobilized cells was markedly decreased (Table 1). Under various NaCl concentrations, the highest H_2 production of 39.09 ± 4.29 μmol H_2 g^{-1} dry weight h^{-1} was obtained in immobilized cells incubated in $BG11_0$ containing 0.5 M NaCl (Table 1). H_2 production by immobilized cells was severely suppressed in cells incubated in $BG11_0$ containing 1 M NaCl (Table 1). Under various concentrations of $MgSO_4 \cdot 7H_2O$, Fe^{3+}, and Ni^{2+}, H_2 production was highest in immobilized cells incubated in $BG11_0$ containing 30 mM $MgSO_4 \cdot 7H_2O$, 4 μM Fe^{3+}, or without Ni^{2+}. Too high concentration of these chemicals decreased H_2 production of immobilized cells (Table 1).

Effect of pH of medium and temperature on H_2 production by immobilized cells

The 3% (*w*/*v*) agar cubes containing 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ supplemented with Turk Island salt solution whose pH was varied from 6 to 10 (pH 7.4 was used as control pH) at 30 °C and subjected to dark fermentative H_2 production assay. The results showed that the highest H_2 production of 39.13 ± 1.15 μmol H_2 g^{-1} dry weight h^{-1} was found in immobilized cells incubated in medium at pH 7.4 under anaerobic dark incubation for 1 day (Fig. 3a). No significant differences with 95% confidence level of H_2 production were found in agar-immobilized cells incubated in medium at pH 7.4 and 8.0. Agar-immobilized cells at pH 6.0 showed the lowest H_2 production (Fig. 3a). To investigate the effect of temperature on H_2 production, agar-immobilized cells were incubated in $BG11_0$ supplemented with Turk Island salt solution (pH 7.4) under darkness at temperature ranging from 20 to 45 °C. The highest H_2 production of 54.30 ± 1.38 μmol H_2 g^{-1} dry weight h^{-1} was observed in immobilized cells incubated at 40 °C under anaerobic dark incubation for 1 day (Fig. 3b). H_2 production was decreased when immobilized cells were incubated at higher or lower temperature than 40 °C (Fig. 3b).

Table 1 H_2 production by immobilized cells of *A. halophytica* incubated in BG11 supplemented with Turk Island salt solution under various $NaNO_3$, NaCl, $MgSO_4 \cdot 7H_2O$, NaCl, Fe^{3+}, and Ni^{2+} concentrations

Composition	Concentration	H_2 production (μmol H_2 g^{-1} dry weight h^{-1})
$NaNO_3$ (mM)	0	39.26 ± 0.38
	0.0176	27.85 ± 1.95
	0.176	5.78 ± 0.96
	1.76	3.19 ± 0.37
	17.6	2.10 ± 1.01
	176	2.43 ± 0.78
NaCl (M)	0.25	18.75 ± 5.19
	0.5	39.09 ± 4.29
	0.75	21.37 ± 1.43
	1	4.41 ± 0.45
$MgSO_4 \cdot 7H_2O$ (mM)	0	38.11 ± 0.94
	1.5	38.94 ± 2.28
	15	39.20 ± 0.51
	30	39.29 ± 2.94
	150	31.62 ± 2.02
Fe^{3+} (μM)	0	37.63 ± 2.91
	0.04	36.74 ± 1.84
	0.4	39.23 ± 1.06
	4	39.37 ± 0.91
	40	39.06 ± 4.31
	400	29.25 ± 1.30
Ni^{2+} (μM)	0	39.25 ± 0.14
	0.1	30.66 ± 2.20
	1	27.84 ± 1.86
	10	25.97 ± 2.14
	100	17.65 ± 1.30

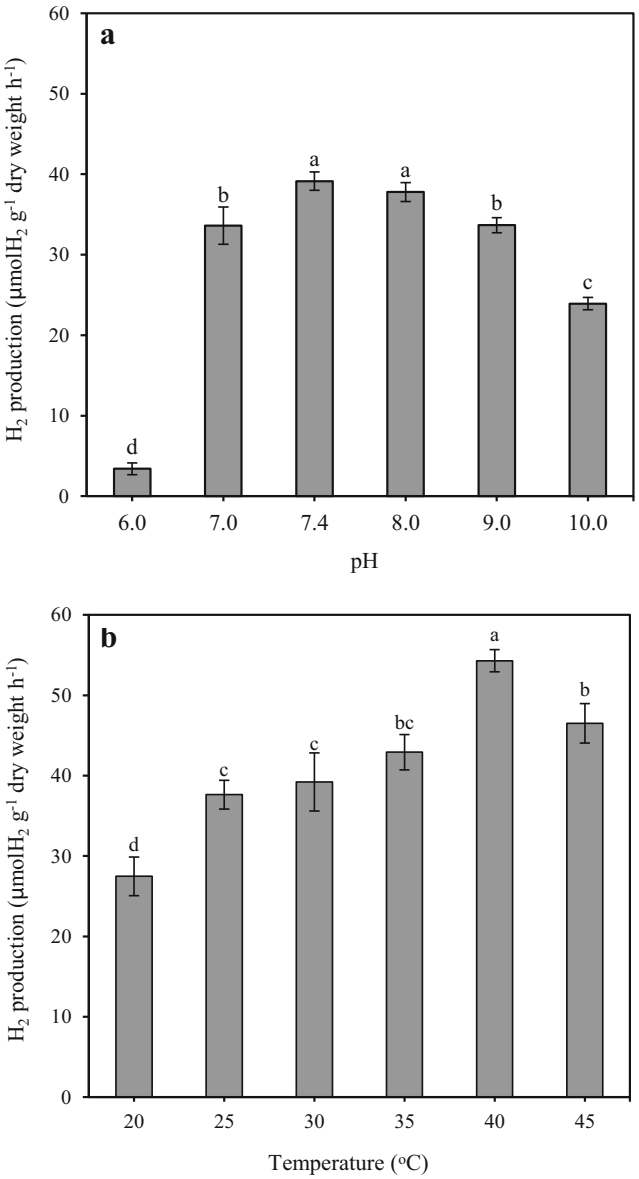

Fig. 3 Effect of pH of medium (**a**) and incubation temperature (**b**) on H_2 production by immobilized cells of *A. halophytica* under N deprivation. Cells were immobilized with 3% (*w*/*v*) agar and final cell concentration was 0.2 mg dry cell weight per mL of gel solution. The agar cubes of immobilized cells were incubated in $BG11_0$ at various pH, 30 °C in the light for 24 h before determination of H_2 production by further incubating the cubes for another 24 h in the dark (**a**). For H_2 production of *A. halophytica* under various temperatures, 3% (*w*/*v*) agar cubes with immobilized cells were incubated in $BG11_0$ at pH 7.4, 30 °C in the light for 24 h before determination of H_2 production by further incubating the cubes at various temperatures for another 24 h in the dark (**b**). Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$

Effect of volumetric size of glass vial, immobilized cells, and headspace on H_2 production by agar-immobilized cells

The cubes of agar-immobilized cells with total volumes of 6 and 36 cm^3 were transferred to 20- and 120-mL glass vials containing 9 and 54 mL of $BG11_0$ supplemented with Turk Island salt solution, respectively (the volumetric ratio of immobilized cells, medium, and headspace was 6:9:5). H_2 production of immobilized cells was measured under dark anaerobic condition at 40 °C for 24 h. The results revealed that immobilized cells in the 120-mL glass vial gave higher H_2 production than those in the 20-mL glass vial (Table 2), indicating the significant effect of container size on H_2 production by immobilized cells. Interestingly, agar-immobilized cells in the 120-mL glass vial showed long-term H_2 production of 2169.87 ± 191.66 μmol H_2 g^{-1} dry weight after 3 days of anaerobic dark incubation, twofold higher than that produced by immobilized cells in the 20-mL glass vial (Table 2). To investigate the effect of immobilized cell volume on H_2 production, cubes of agar-

immobilized cells with total volumes of 15, 24, 36, and 60 cm^3 were transferred to 120-mL glass vials and medium was added into vials with the different volumes in order that headspace volume was fixed at 30 mL. The results showed that lower immobilized cell volume gave higher H_2 production (Table 2). The 15-cm^3 cube of agar-immobilized cells gave the highest H_2 production of 135.54 ± 1.92 μmol H_2 g^{-1} dry weight h^{-1} with the maximum H_2 production yield of 4410.88 ± 56.84 μmol H_2 g^{-1} dry weight at 3 days of anaerobic dark incubation (Table 2). To study the effect of headspace volume on H_2 production, immobilized cells with a total volume of 36 cm^3 were transferred to a 120-mL glass vial and medium was added into vials with the different volumes in order that headspace volume was different. It was shown that at the same volume of immobilized cells, the differences of headspace volume affected H_2 production. The results showed that an increase in headspace volume led to an increase of both H_2 production rate and H_2 production yield of immobilized cells (Table 2).

Cycle of H_2 production by immobilized cells

H_2 production by agar-immobilized cells and free cells was investigated for three cycles. H_2 production of agar-immobilized cells was approximately two- to threefold higher than that of free cells (Fig. 4a-d). In each cycle of H_2 production, agar-immobilized cells showed the highest H_2 production after 24 h of anaerobic dark incubation (Fig. 4a-d). After that, little or no increase of H_2 production was detected. When the immobilized cells were repeatedly used in the second and third cycles, H_2 production was decreased (Fig. 4a). However, replacement of 30, 50, and 100% of the immobilized cells after the first cycle with the same respective volume of newly fresh immobilized cells resulted in the stability of H_2 production by immobilized cells in the second and third cycles (Fig. 4b-d). The replacement with all newly fresh immobilized cells gave the maximum H_2 production in cycles 2 and 3 comparable with that found in cycle 1 (Fig. 4d). Interestingly, 50% of immobilized cell replacement caused similar H_2 production in cycle 2 as compared with that in cycle 1 (Fig. 4c).

Discussion

In this study, H_2 production of unicellular halotolerant cyanobacterium *A. halophytica* immobilized with the three support materials (agar, agarose and κ-carrageenan) was higher than that of free cells (Fig. 1). The immobilization of cells on support materials enables the cells to separate from a liquid phase, thus protecting bidirectional hydrogenase of the cells from O_2, a strong cyanobacterial hydrogenase inhibitor, in an external environment. As a result of uninhibited hydrogenase activity, an increase of H_2 production could be observed.

In our immobilized system we used $BG11_0$ supplemented with Turk Island salt solution as a solution for suspension of cyanobacterial cells and solubility of agar, agarose and κ-carrageenan gels. This $BG11_0$ medium lacking nitrogen sources has been shown as an optimal medium for maximizing H_2 production by *A. halophytica* (Taikhao et al. 2013). Among all support materials cells immobilized with agar gave the highest H_2 production compared with cells immobilized with other support materials (Fig. 1). In addition, agar gel showed a high stability when solubilized in $BG11_0$ supplemented with Turk Island salt solution. It has been reported that agar had an advantage with regard to gel solidity and stability under both alkaline and acidic conditions, and even

Table 2 Effect of sizes of glass vial, headspace volume, and immobilized cells volume on H_2 production by immobilized cells of *A. halophytica*

Volumetric size (mL)				Maximum H_2 production rate (μmol H_2 g^{-1} dry weight h^{-1})	Maximum H_2 production yield (μmol H_2 g^{-1} dry weight)
Glass vial	Immobilized cells	Medium	Headspace		
20	6	9	5	63.03 ± 3.10	1034.72 ± 32.16
120	36	54	30	67.44 ± 9.27	2169.87 ± 191.66
120	36	69	15	38.96 ± 3.78	1428.82 ± 8.96
120	36	66	18	51.97 ± 6.15	1644.28 ± 212.29
120	36	64	20	57.45 ± 4.47	1847.13 ± 100.76
120	36	60	24	63.97 ± 1.06	1965.45 ± 108.63
120	36	54	30	67.44 ± 9.27	2169.87 ± 191.66
120	36	44	40	77.03 ± 1.46	2209.15 ± 19.38
120	54	36	30	55.94 ± 0.99	1868.30 ± 120.23
120	36	54	30	67.44 ± 9.27	2169.87 ± 191.66
120	24	66	30	117.44 ± 0.72	3497.03 ± 131.76
120	15	75	30	135.54 ± 1.92	4410.88 ± 56.84

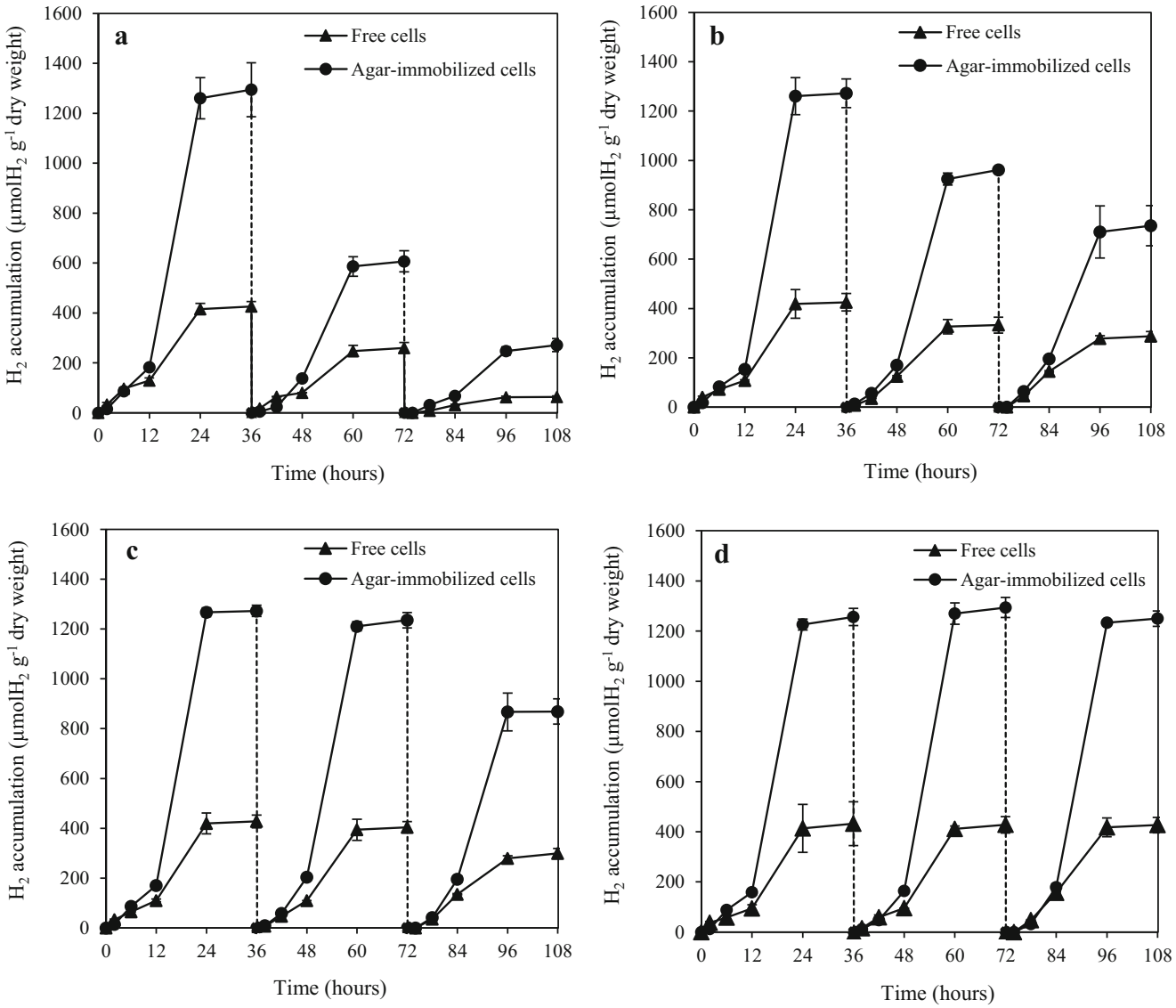

Fig. 4 H_2 production cycles by the cubes with agar-immobilized cells of *A. halophytica* under N deprivation. Cells were immobilized with 3% (*w*/*v*) agar and final cell concentration was 0.2 mg dry cell weight per mL of gel solution. The cubes were incubated at 30 °C in the light for 24 h before determination of H_2 production in the dark for three cycles (36 h for each cycle). After finishing each H_2 production cycle, the old medium was removed and replaced with a fresh medium. The cubes of immobilized cells were used in another two cycles of H_2 production without a replacement with fresh cubes of immobilized cells (**a**) and with 30% (**b**), 50% (**c**), and 100% (**d**) replacement with fresh agar cubes in cycles 2 and 3. The fresh agar cubes with immobilized cells were earlier adapted in N-deprived medium at 30 °C under the light for 24 h. Free cell experiments were done in the same manner but without a procedure of cell immobilization

in the presence of salts, minerals, and chelating agents (Scott 2012). For carrageenan gel formation, NaCl in the medium might decrease the strength of carrageenan gel as previously reported (Nguyen et al. 2014), resulting in a low stability of carrageenan gel. Likewise, agarose gel also had lower stability than agar gel (Semenchuk et al. 2000). It is highly likely that cells immobilized in a low strength or less stable gel may leak from the gel, leading to a decreased H_2 production. In this study, agar-immobilized cells showed higher H_2 production than alginate-immobilized cells as previously reported (Pansook et al. 2016). Our results were consistent with a previous study showing that the average H_2 production by 1.5% (*w*/*v*) agar-immobilized blocks of non-diazotrophic cyanobacterium *Oscillatoria subbrevis* strain 111 was slightly higher than that by 1.5% (w/*v*) alginate-immobilized cells (Kumar et al. 1991). On the other hand, in filamentous cyanobacterium *Lyngbya perelegans*, H_2 production by 2% (*w*/*v*) alginate-immobilized cells was slightly higher than that by 2% (*w*/*v*) agar-immobilized cells (Anjana and Kaushik 2014). Moreover, 2% (*w*/*v*) carrageenan-immobilized cells of heterocystous cyanobacterium *Anabaena* N-7363 had slightly higher H_2 production than 2.5% (*w*/*v*) agar-immobilized cells and 3% (*w*/*v*) alginate-immobilized cells (Karube et al. 1986). Therefore, it was suggested that the suitable type of support

material for H_2 production by immobilized cells is dependent on the species of cyanobacteria.

The 3% (*w*/*v*) agar-immobilized cells of *A. halophytica* gave the highest H_2 production compared with 1, 1.5, 2 and 2.5% (*w*/*v*) agar-immobilized cells (Fig. 2 a). The results indicated that higher agar concentrations led to higher level of polymerization, thus resulting in a decrease of gel porosity. The reduced pore sizes of 3% (*w*/*v*) agar gel can hinder the access of O_2 existing in the system. Therefore, bidirectional hydrogenase would be more active due to a decrease of O_2. This led to an increase of H_2 production in cells immobilized with high agar concentrations. In addition, an increase of agar concentration could enhance cell survival and mechanical stability of gel (Seol et al. 2011). Our results indicate that the suitable concentration of support material for immobilization is an important contributing factor for the improvement of H_2 production by immobilized cells.

Further investigation was performed to study the effect of cell concentration on H_2 production by 3% (*w*/*v*) agar-immobilized *A. halophytica*. Agar-immobilized cells containing 0.2 mg dry cell weight per mL of gel solution gave the highest H_2 production rate (Fig. 2b). The lower H_2 production rate by agar-immobilized cells of *A. halophytica* with higher cell concentrations might also be due to the diffusional problems of essential nutrients or minerals, thus reducing the ability of entrapped cells for effective exposure to available nutrients. This may lead to the reduction in metabolic activity of cells with the consequence of the reduced electrons flow towards bidirectional hydrogenase, resulting in a decrease of H_2 production. In addition, higher cell concentrations in agar gel might lead to the competition of substrate binding and also an insufficient amount of substrate or cofactor. The high cell concentration of immobilized cyanobacterium *L. perelegans* has been reported to decrease the mechanical stability of immobilization system, further leading to the increased porosity which allowed for easier diffusion of O_2 into the gel matrix that caused an inhibition of hydrogenase activity (Anjana and Kaushik 2014).

Not only the type of support material, but its volumetric size could also affect H_2 production of the immobilized cells. The volumetric sizes higher than 0.125 cm^3 showed a decrease of H_2 production due to the difficulty of H_2 diffusion in or out of the gel (Fig. 2c). Nevertheless, it should be noted that the optimal volumetric size of support material for high H_2 production can vary depending on cyanobacterial species (Karube et al. 1986; Kumar et al. 1991; Rashid et al. 2009; Anjana and Kaushik 2014).

Immobilized cells of *A. halophytica* incubated in nitrate-free BG11 ($BG11_0$) supplemented with Turk Island salt solution showed the highest H_2 production (Table 1). Under N deprivation, cyanobacteria reduce protein synthesis but rather accumulate glycogen within the cells. When cells are under anoxic condition, storage glycogen as a source of electron donor is degraded and the released electrons are used for H_2 evolution by bidirectional hydrogenase activity (Troshina et al. 2002). Previous studies on H_2 production by immobilized cells of cyanobacteria such as non-heterocystous filamentous *Plectonema boryanum*, unicellular *Gloeocapsa alpicola* CALU 743, and unicellular *Synechocystis* sp. PCC 6803 were also performed under nitrogen deprivation (Sarkar et al. 1992; Serebryakova and Tsygankov 2007; Touloupakis et al. 2016). The effect of nitrogen deprivation on H_2 production by these immobilized cells was similar to that reported in free cells of several cyanobacterial strains such as *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui 1981), *G. alpicola* (Serebryakova et al. 1998; Troshina et al. 2002), *Arthrospira maxima* (Ananyev et al. 2008), *A. halophytica* (Taikhao et al. 2013), and *Anabaena siamensis* TISTR 8012 (Taikhao and Phunpruch 2017).

The composition and concentration of some nutrients and mineral ions in medium play an important role in H_2 production of cyanobacteria. H_2 production by immobilized cells of *A. halophytica* was highest in $BG11_0$ containing either 0.5 M NaCl, 30 mM $MgSO_4 \cdot 7H_2O$, 4 μM Fe^{3+} or 0 μM Ni^{2+} (Table 1). These concentrations of each compound were present in BG11 supplemented with Turk Island salt solution and were optimal for H_2 production by free cells of *A. halophytica* (Taikhao et al. 2013). Due to the typical halophilic characteristic of *A. halophytica*, NaCl is required for intracellular metabolism and cell survival including optimal H_2 production. In marine cyanobacterium *Lyngbya* sp. strain 108, the highest H_2 production was observed in cells grown in medium containing 3% (*w*/*v*) or 0.5 M NaCl (Kuwada and Ohta 1989). Sulfur is a constituent of amino acids, cysteine and methionine, that are very important for the function of many enzymes and proteins, especially D1 protein which is essential for photosystem II. In this study, the sulfur deprivation did not promote H_2 production by these immobilized cells. Since we focused on the dark fermentative H_2 production by immobilized cells of *A. halophytica*, the lowering D1 protein as affected by sulfur deprivation played no role in dark fermentative H_2 production. Iron and nickel ions are bimetallic cofactors of NiFe-hydrogenase (Peters et al. 2015). The availability of iron facilitates more electron transport towards hydrogenase to evolve H_2 (Lin and Stewart 1997). However, in this study, an addition of nickel in the medium did not promote H_2 production by immobilized cells of *A. halophytica*, possibly due to the toxicity of nickel to cyanobacterial cells (Babich and Stotzky 1983).

The headspace volume of glass vial was found to affect H_2 production by agar-immobilized cells. When the same sizes of glass vial and immobilized cells were used, an increase in the headspace volume caused an increase in H_2 production (Table 2), confirming that H_2 production was dependent on the headspace volume. This increased H_2 production could be due to the equilibrium of the bidirectional hydrogenase

reaction favoring the production rather than the uptake when there is a larger headspace volume. These results were consistent with those in the three microalgal strains of *Chlamydomonas reinhardtii* (CC-125, CC-4169, and CC-4170) (Altimari et al. 2014). Interestingly, H_2 production rate and H_2 accumulation were maximum in immobilized cells with their lowest volumetric size (Table 2). This might be ascribed to the high efficiency of H_2 diffusion when using a small volume of immobilized cells. This result was similar to that of the above study on the effect of cell concentration on H_2 production.

Finally, to sustain H_2 production, the re-use of immobilized cells of *A. halophytica* was determined for three cycles. H_2 production of immobilized cells was evidently higher than that of free cells in all three cycles (Fig. 4). In addition, the repeated use of immobilized cells in all three cycles led to the lower H_2 yield in cycles 2 and 3 (Fig. 4a). It is not clear as to what caused the decrease of H_2 production by the free cells after the first cycle. It might be possible that cells were inactive and had decreasing amounts of the accumulated carbohydrates and reducing powers after 36 h of dark anaerobic incubation for the first cycle of H_2 production. The replacement by fresh immobilized cells was a choice for sustainable H_2 production by immobilized cells of *A. halophytica.* The 50% replacement by fresh immobilized cells gave similarly high H_2 yield in cycle 2 to that found in cycle 1 (Fig. 4c). Much effort has been made to promote and sustain H_2 production from immobilized cells incubated for several cycles. Until now, it has not been successful yet. This is probably due to the high O_2 sensitivity of bidirectional hydrogenase of *A. halophytica* and limitation of support materials used for immobilization. H_2 production of immobilized cells for many cycles has been previously studied in several species of cyanobacteria. Alginate-immobilized cells of wild type and Δ*hupL* mutant of *Calothrix* 336/3 prolonged H_2 production over several cycles whereas suspension cultures reduced significantly H_2 production after the first cycle (Leino et al. 2009). Agar-immobilized cells of *Microcystis aeruginosa* could produce H_2 up to three cycles and H_2 was stably generated for more than 40 h in each cycle (Rashid et al. 2009). Notably, *Gloeocapsa alpicola* CALU 743 immobilized in glass fiber produced H_2 in a long-term cyclic regime and provided relatively stable H_2 production over a period of not less than 20 days (Serebryakova and Tsygankov 2007).

In conclusion, cell immobilization clearly enhances H_2 production efficiency in the unicellular halotolerant cyanobacterium *A. halophytica*. The type and concentration of support material for immobilization including cell concentration influence H_2 production. $BG11_0$ supplemented with Turk Island salt solution contains suitable amount of nutrients and minerals for H_2 production in immobilized cells. The optimal pH and incubation temperature were 7.4 and 40 °C, respectively. Higher size of container and headspace volume increases H_2 production whereas higher volume of immobilized cells decreases H_2 production. H_2 production by agar-immobilized cells becomes lower after repeated use. Lastly, the replacement with fresh immobilized cells could help sustain H_2 production in this cyanobacterial strain.

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