



Enhanced hydrogen production by *Nostoc* sp. CU2561 immobilized in a novel agar bead

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Abstract

A large number of microalgae isolated from Thailand were screened for their hydrogen production capacity. The selected highly efficient microalga, identified as *Nostoc* sp. CU2561, was investigated for the conditions under which the cells had maximal hydrogen production rate. *Nostoc* sp. CU2561 showed highest hydrogen production rate when grown in BG11 medium deprived of nitrogen and sulfur (BG11-N-S). To further improve hydrogen production, newly invented agar beads were used as matrix for cell immobilization. The highest hydrogen production rate by 1.5% (w/v) agar bead immobilized cells was obtained using cell concentration of 0.125 mg dry wt mL⁻¹. Agar bead-immobilized cells showed superior hydrogen production rate, 1.5-fold higher when compared with agar cube-immobilized cells, and 5- and 10-fold higher when compared with those in alginate bead-immobilized and alginate bead-suspended cells, respectively. Supplementation of 0.5% (w/v) fructose increased hydrogen production rate of agar bead-immobilized cells approximately 1.7-fold, whereas the reducing agent β-mercaptoethanol increased hydrogen production rate by about 8.2-fold. Overall, *Nostoc* sp. CU2561 immobilized in agar bead showed the highest hydrogen production rate when incubated in BG11-N-S containing 5 mM β-mercaptoethanol with the highest hydrogen production rate of 18.78 ± 1.44 μmol H₂ mg⁻¹ chl *a* h⁻¹. In addition, agar bead-immobilized cells could continuously produce hydrogen for 3 cycles. The hydrogen production by immobilized cells could be prolonged up to 120 h during the first cycle. This study provides a potential of new immobilization strategy using cyanobacteria immobilized in agar bead for hydrogen production which can be applied to scale up at industrial level in the future.

Keywords Hydrogen production · Immobilization · Agar bead · Cyanobacteria · *Nostoc* sp. CU2561

Introduction

With an increase of population coupled with the advancement of technology and economic growth, the world is facing energy depletion crisis. The development of biofuel production is therefore needed to overcome current challenges such as economic and environmental problems. Hydrogen is an alternative

renewable energy which can be produced using organisms or other biological systems which can capture sunlight and convert it to chemical energy (Baker and Paul 2017). Hydrogen is a potentially sustainable energy source in the future due to its abundance and non-polluting nature (Mathews and Wang 2009). Hydrogen derived from biological sources (biohydrogen) is more environmentally friendly than that derived by chemical processes. Biohydrogen production from microalgae is advantageous because CO₂ fixation and nutrient removal from water can be accomplished simultaneously with H₂ production (Melis et al. 2000; Kim et al. 2008). Photosynthetic microorganisms such as cyanobacteria can generate hydrogen from solar energy and water (Tamagnini et al. 2007). Cyanobacteria produce hydrogen through N₂ fixation process with nitrogenase activity and through photosynthetic process with bidirectional hydrogenase activity (Benemann 2000; Bothe et al. 2010; Khetkorn et al. 2017).

The bidirectional hydrogenase accepts electrons from two processes. First, solar energy is captured at PSII and then followed by water splitting. The obtained electrons are transferred through PSI to reduce ferredoxin. Finally, the proton

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will accept electrons from the reduced ferredoxin to form hydrogen catalyzed by bidirectional hydrogenase. In the second process, the storage glycogen is catabolized and the resulting electrons are shuttled to bidirectional hydrogenase for biohydrogen production (Ananyev et al. 2008).

Hydrogen production of N_2 -fixing cyanobacteria depends enormously on nitrogenase activity. Cells grown with supplemented nitrogen sources had reduced nitrogenase activity with a subsequent decrease of hydrogen production (Madamwar et al. 2000; Khetkorn et al. 2010). Upon N deprivation, heterocyst differentiation is stimulated leading to an increase in nitrogenase activity and an increase of hydrogen production (Khetkorn et al. 2010). On the other hand, S deprivation also has been shown to increase hydrogen production in a green microalga as a result of a decrease of PS II-mediated O_2 evolution which was caused by the lack of sulfur, an important component of D1 protein of PS II (Melis et al. 2000).

There have been several attempts to improve hydrogen production using cell immobilization. The immobilization of cyanobacteria for hydrogen production has been extensively studied using two types of support materials, namely agar (Kayano et al. 1981; Philips and Mitsui 1986; Rashid et al. 2009; Anjana and Kaushik 2014; Taikhao and Phunpruch 2017; Pansook et al. 2019; Wutthithien et al. 2019) and alginate (Leino et al. 2012; Kosourov et al. 2017; Maswana et al. 2018). Agar is a polysaccharide extracted from red algae which consists of heterogeneous colloidal polysaccharide complex of agarose and agarpectin with alternating α -(1 \rightarrow 3) linkages and β -(1 \rightarrow 4) linkages, whereas alginate is an unbranched binary copolymer of β -D-mannuronic acid and α -L-guluronic acid. *Aphanothece halophytica* cells immobilized in an agar cube have been reported to have increased hydrogen production efficiency with prolonged hydrogen production for several cycles (Pansook et al. 2019). Similarly, the hydrogen production of *Lyngbya perelegans* immobilized in an agar cube and alginate bead had a 2–4-fold higher hydrogen production than that of free cells (Anjana and Kaushik 2014). Immobilized cells of the cyanobacterium *Microcystis aeruginosa* in an agar cube were able to stably generate hydrogen for more than 40 h in each cycle during three repeated cycles of alternating photosynthesis and hydrogen production (Rashid et al. 2009). Immobilization of *Oscillatoria* sp. Miami BG 7 in agar with rectangular shape improved the hydrogen production rate with sustained production for 3 weeks (Philips and Mitsui 1986). So far, immobilization of cyanobacteria for hydrogen production has been reported using alginate (beads and films) and agar including agarose and κ -carrageenan (cube and rectangular shape) (Rashid et al. 2009; Leino et al. 2012; Anjana and Kaushik 2014; Taikhao and Phunpruch 2017; Kosourov et al. 2017; Maswana et al. 2018; Pansook et al. 2019; Wutthithien

et al. 2019). To our knowledge, there has been no work on the use of agar beads as a matrix for cyanobacterial hydrogen production.

Materials and methods

Sampling and species identification

A total of 290 isolates from several fresh water sources and rice paddy fields in central part and eastern part of Thailand were plated on agar prepared with BG11 medium and left under light for several weeks until microalgae colonies were detected. The single colony was picked and streaked on a new agar plate. Restreaking continued until the contaminant was eliminated. All purified isolates grown in BG11 medium were individually determined for their hydrogen production capacity during 1 h incubation using a gas chromatograph. The selected cyanobacterial species was identified using 16S ribosomal RNA gene-specific primers designed from the conserved region of 16S ribosomal RNA sequences in the NCBI nucleotide database from seven different cyanobacterial species. Genomic DNA was extracted by isoamyl:chloroform method (Vaidyanathan et al. 2012). The 16S ribosomal RNA sequence was compared with sequences from Gen Bank using the Blast program (Altschul et al. 1997). The morphology of the cyanobacterium was observed using a light microscope. The isolated cyanobacterium was identified as *Nostoc* sp. CU2561.

Cyanobacterial growth and cultivation conditions optimization

Nostoc sp. CU2561 culture was kept in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (Rippka et al. 1979). The culture was shaken on a rotary shaker at 160 rpm at 30 °C under a continuous light intensity of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 7–14 days. *Nostoc* sp. CU2561 growth was monitored by measuring optical density at 730 nm.

To study the effect of nitrogen deprivation (BG11-N) or sulfur deprivation (BG11-S) or nitrogen and sulfur deprivation (BG11-N-S) on hydrogen production, the 7-day cells grown in BG 11 (for testing BG11-S conditions) and grown in BG11-N (for testing BG11-N and BG11-N-S conditions) were first adapted in each respective tested medium under light at 30 °C for 24 h. The pellet, harvested by centrifugation at 5500 rpm for 10 min at room temperature, was washed twice and resuspended with the fresh respective medium before subjecting to hydrogen measurement after 8h incubation. BG11-N is BG11 without NaNO_3 and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ is replaced with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, whereas BG11-N-S is BG11-N in which $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ is replaced with $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ is

replaced with CuCl_2 and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ is replaced with $\text{ZnCl}_2 \cdot 2\text{H}_2\text{O}$, all in equal molarity.

PCR amplification, sequencing, and phylogenetic analysis using 16S rRNA gene sequences

The forward primer was 5'AGAGTTTGATCCTGGCTCA' and the reverse primer was 5'ACGGCTACCTTGTTACGACT3'. The PCR program was 3 min at 94 °C followed by 31 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The PCR reaction mixtures were analyzed by electrophoresis using 0.8% agarose gels. The amplification product was purified with the PCR Purification Kit (GenepHlow Gel/PCR Kit, Taiwan) and sent for sequencing by Pacific Science Sequencing Service with the same forward primer and reverse primer used in amplification reactions (Sciuto and Moro 2016). The 16S ribosomal RNA gene sequence was compared with sequences from the database of Gen Bank (Hellberg et al. 2013) and the sequence was used for alignment (CLUSTAL-X) prior to Bootstrap NJ phylogenetic tree analysis.

Cyanobacterial cell immobilization

The cells at the logarithmic growth phase were harvested by centrifugation at 5500 rpm for 10 min at room temperature. The pellet was washed twice and resuspended in the same fresh medium. The cell suspension was added to the sterile sodium alginate solution to make the final alginate concentration of 4% (w/v) and mixed homogeneously at room temperature. The reaction mixture was dropped with syringe into sterile 2% (w/v) CaCl_2 solution (Maswana et al. 2018). For immobilized cells in agar bead, the cell suspension was added to the sterile agar solution at 40–45 °C to make the final agar concentration and cell concentration as desired. The mixture was dropped with syringe into a sterile soybean oil cooled in ice bath. Each agar bead had the diameter of 0.4 cm. In addition, cells were also immobilized in agar cube where the solidified agar with immobilized cells was cut into cube shape ($0.5 \times 0.5 \times 0.5$ cm).

Optimization of cell immobilization for improving hydrogen production rate

Nostoc sp. CU2561 was pre-grown in BG11-N medium for 7 days. Cells were washed and transferred to BG11-N-S medium and incubated under the light for 24 h. Then *Nostoc* sp. CU2561 cells were immobilized in 4% (w/v) sodium alginate bead, 1.5% (w/v) agar beads and 1.5% (w/v) agar cube, at cell concentration of 0.25 mg mL^{-1} and transferred to a glass vial before detecting H_2 production using a gas chromatograph.

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

Nostoc sp. CU2561 was grown in BG11-N medium for 7 days. Cells were washed and transferred to BG11-N-S medium and incubated under the light for 24 h. The *Nostoc* sp. CU2561 cells were immobilized in 1.5% (w/v) agar beads, with cell concentration ranging from 0.031 – 10 mg mL^{-1} . In addition, effect of agar concentration ranging from 0.5 to 4.5% (w/v) using cell concentration at 0.125 mg mL^{-1} was also studied. The immobilized cells of *Nostoc* sp. CU2561 were transferred to a glass vial before detecting H_2 production.

Effect of sugar sources and reducing agents on H_2 production by agar-immobilized cells

Nostoc sp. CU2561 cells were grown in BG11-N medium for 7 days. Cells were washed and transferred to BG11-N-S and BG11-N-S supplemented with 0.5% (w/v) of various kinds of sugar. In addition, effect of reducing agent on H_2 production by agar-immobilized cells in BG11-N-S was also done by supplementation with 5 mM of dithiothreitol, β -mercaptoethanol, and methylviologen followed by incubation for 24 h under a light intensity of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Cell suspension in the same respective medium was then immobilized in 1.5% (w/v) agar beads with cell concentration of 0.125 mg mL^{-1} before transferring to a glass vial for H_2 production determination.

Measurement of hydrogen production

Suspended or immobilized cells were transferred to a gas-tight glass vial. The glass vial was capped with a rubber septum and bubbled with argon gas for 15 min (Taikhao and Phunpruch 2017). The 13.5-mL vials were incubated under a light intensity of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at room temperature for 8 h before H_2 measurement. There was no detectable H_2 at the start of the incubation. For free cell experiments, 5-mL cell suspension was used leaving 8.5 mL as a head space, whereas in immobilized cell experiments, the respective medium was added to the vial so as to allow 8.5 mL as a head space; the ratio of agar bead to liquid medium was 1:1.8. H_2 in the head space of the gas-tight vial was determined after 8 h incubation, during which the linear increase in the amount of hydrogen was observed at 2, 4, and 8 h, under a light intensity of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ using a gas chromatograph with a Molecular Sieve 5A 60/80 mesh column (Perkin Elmer, USA) equipped with a thermal conductivity detector (Baebprasert et al. 2010). The hydrogen production rate is expressed as $\mu\text{mol H}_2$ per mg chlorophyll a per h (Khetkorn et al. 2010). Chlorophyll *a* was extracted from the cell with 90% (v/v) methanol at room temperature. Chlorophyll *a* concentration

was determined spectrophotometrically at 665 nm as previously described (Mackinney 1941).

Sustaining H₂ production by agar bead-immobilized cells

Nostoc sp. CU2561 cells were immobilized in 1.5% (w/v) agar at cell concentration of 0.125 mg mL⁻¹ with different media, i.e., BG11-N (Agar bead), BG11-N-S (Agar cube), and BG11-N-S (Agar bead) and incubated in each respective medium for various times. At the end of each cycle where no increase of hydrogen production was detected, the old medium was removed and replaced with the new fresh medium before measuring hydrogen production as described above.

Statistical analysis

All experiments were performed in triplicate and the average values are reported. The statistical differences were determined according to Duncan's multiple range test using program IBM SPSS statistics version 22 at $p < 0.01$. In all figures, different letters on columns indicate a significant difference with the lowest value starting with the letter a, according to Duncan's multiple range test at $p < 0.01$.

Results

Screening microalgae for hydrogen production

The hydrogen production capacity of all the 290 isolates of microalgae was determined and the results are shown in Fig. S1. The isolate number 59 from Singburi province had highest production rate of 0.013 $\mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$. The identified *Nostoc* sp. CU2561 cells consisted of filaments and the specialized cells, heterocysts (Fig. 1).

PCR and phylogenetic tree

The 16S rRNA sequences revealed by phylogenetic tree (Fig. 2) enable the identification of the cyanobacterium as *Nostoc* sp. CU2561. The phylogenetic tree shows several groups of cyanobacteria. Although the phylogenetic tree does not classify *Nostoc* into a single group, presumably, the selected cyanobacterium should be *Nostoc* sp. because each group in the tree has a very near distance. Therefore, the slight differences in 16S rRNA sequences may cause separation into different groups. The 16S rRNA sequences of phylogenetic tree determination identified the studied cyanobacterium as *Nostoc* sp. CU2561 clustered with other *Nostoc* sp. with the accession number AM71154.1, KM019925, LC19059.1, KU645906.1, AY742452.1, and MG272378.1.

Effect of sulfur deprivation on H₂ production

Sulfur deprivation in *Nostoc* sp. CU2561 increased hydrogen production by about 2-fold when the cells were pre-grown for 7 days in either BG11 (compare between BG11 and BG11-S conditions) or BG11-N (compare between BG11-N and BG11-N-S conditions) medium (Fig. 3). It is interesting to note that when comparing between BG11 and BG11-N conditions, nitrogen deprivation was more effective than sulfur deprivation in promoting hydrogen production with about a 5-fold increase in the production. The combined effect of both nitrogen and sulfur deprivation (BG11-N-S) resulted in the highest H₂ production rate of $0.13 \pm 0.01 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$ and this condition was used in further experiments to enhance hydrogen production.

Cell immobilization for improving hydrogen production rate

The hydrogen production by *Nostoc* sp. CU2561 cells immobilized in 4% (w/v) sodium alginate beads, 1.5% (w/v) agar beads, 1.5% (w/v) agar cube, and by free cells is shown in Fig. 4. *Nostoc* sp. CU2561 had the highest hydrogen production rate when immobilized in 1.5% (w/v) agar beads. Hydrogen production rate in agar bead-immobilized *Nostoc* sp. CU2561 was $1.26 \pm 0.06 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$, 1.5-, 5-, and 9-fold higher than that in agar cube-immobilized, alginate bead-immobilized, and free cells, respectively. Thus, the agar bead immobilized cells were used for the optimization of hydrogen production in further experiments.

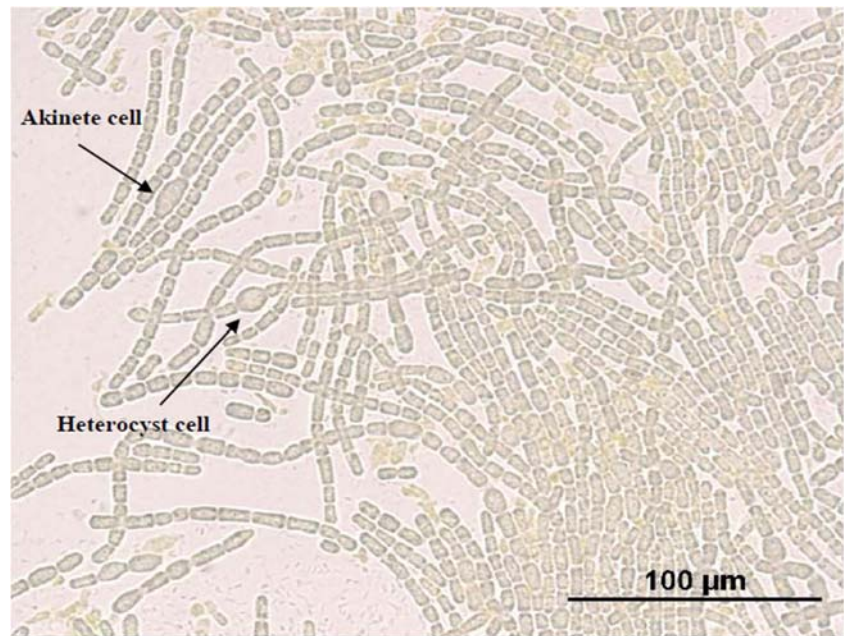
Effect of cell concentration on H₂ production by agar bead-immobilized cells

Hydrogen production rates by agar bead-immobilized *Nostoc* sp. CU2561 at different cell concentrations were determined. Immobilized *Nostoc* sp. CU2561 showed the highest hydrogen production rate of $2.28 \pm 0.06 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$ when immobilized with 0.125 mg cell dry wt mL⁻¹ agar (Fig. 5). Consequently, this cell concentration was used for the optimization of hydrogen production in further experiments.

Effect of agar concentration on H₂ production by agar bead-immobilized cells

The immobilized *Nostoc* sp. CU2561 cells in agar beads at different agar concentrations was optimized by changing the agar concentration at 0.5, 1.5, 2.5, 3.5, and 4.5% (w/v) agar bead before transferring to a glass vial for detecting H₂ production. *Nostoc* sp. CU2561 showed an increase of hydrogen production rate with increasing agar concentration up to 4.5% (w/v) (Fig. 6). However, at an agar concentration of 1.5% (w/v), the bead was of perfect spherical shape while at higher

Fig. 1 Cell morphology of *Nostoc* sp. CU2561 observed under a light microscope



than 2.5% (w/v), the bead became harder and tailing which was clearly visible at 4.5% (w/v). Therefore, immobilization of *Nostoc* sp. CU2561 in 1.5% (w/v) agar beads producing $2.36 \pm 0.06 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$ was chosen for long-term and stable production of hydrogen, due to a durable gel strength of spherical agar beads. Therefore, agar concentration of 1.5% (w/v) was used for the optimization of hydrogen production in further experiments.

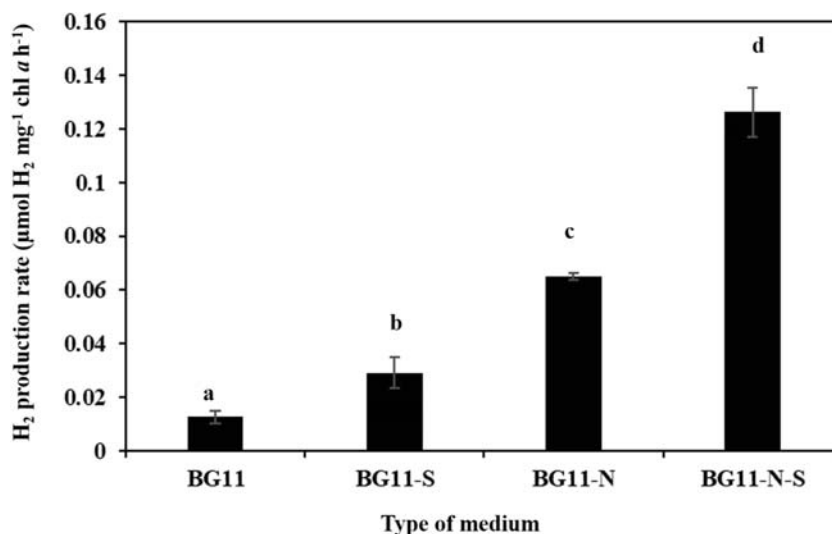
Effect of different sugar sources on H₂ production by agar bead-immobilized cells

Nostoc sp. CU2561 cells were grown in BG11-N medium for 7 days and transferred to BG11-N-S and BG11-N-S media supplemented with 0.5% (w/v) of various kinds of sugar. Cells immobilized in 1.5% (w/v) agar beads showed the highest hydrogen production rate of $3.96 \pm$



Fig. 2 Phylogenetic tree based on 16S rRNA sequences of *Nostoc* sp. CU2561 and 26 other cyanobacteria species. The number at the end of the name is accession number available in the NCBI database

Fig. 3 H₂ production rate by *Nostoc* sp. CU2561 under sulfur and nitrogen deprivation. Cells were adapted in BG11, BG11-S, BG11-N, and BG11-N-S media for 24 h before determination of H₂ production by incubation in the same respective medium for 8 h under light condition. Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters



0.13 μmol H₂ mg⁻¹ chl a h⁻¹ when incubated in BG11-N-S medium containing 0.5% (w/v) fructose, about a 1.7-fold higher than that incubated in the sugar-free BG11-N-S medium (Fig. 7).

Effect of reducing agent on H₂ production by agar bead-immobilized cells

Nostoc sp. CU2561 cells were transferred to BG11-N-S and BG11-N-S supplemented with 5 mM of dithiothreitol, β-mercaptoethanol, and methylviologen. Cells were then immobilized in 1.5% (w/v) agar beads. *Nostoc* sp. CU2561

showed the highest hydrogen production rate of 18.78 ± 1.44 μmol H₂ mg⁻¹ chl a h⁻¹ when incubated in BG11-N-S containing 5 mM of β-mercaptoethanol, about a 8.2-fold higher than that incubated in the reducing agent-free BG11-N-S medium (Fig. 8).

Comparison of the maximum hydrogen production from immobilized cells of *Nostoc* sp. CU2561 with other immobilized cyanobacterial strains

The results in Table 1 indicate that the maximum hydrogen production rate from *Nostoc* sp. CU2561 immobilized

Fig. 4 Hydrogen production rate by *Nostoc* sp. CU2561 immobilized in different types of matrix support. Cells were immobilized in 4% (w/v) sodium alginate, 1.5% (w/v) agar beads, and 1.5% (w/v) agar cube, at cell concentration of 0.25 mg mL⁻¹ before transferring to a glass vial for determination of H₂ production. Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters. The structural shape of each gel matrix is also shown in the panel

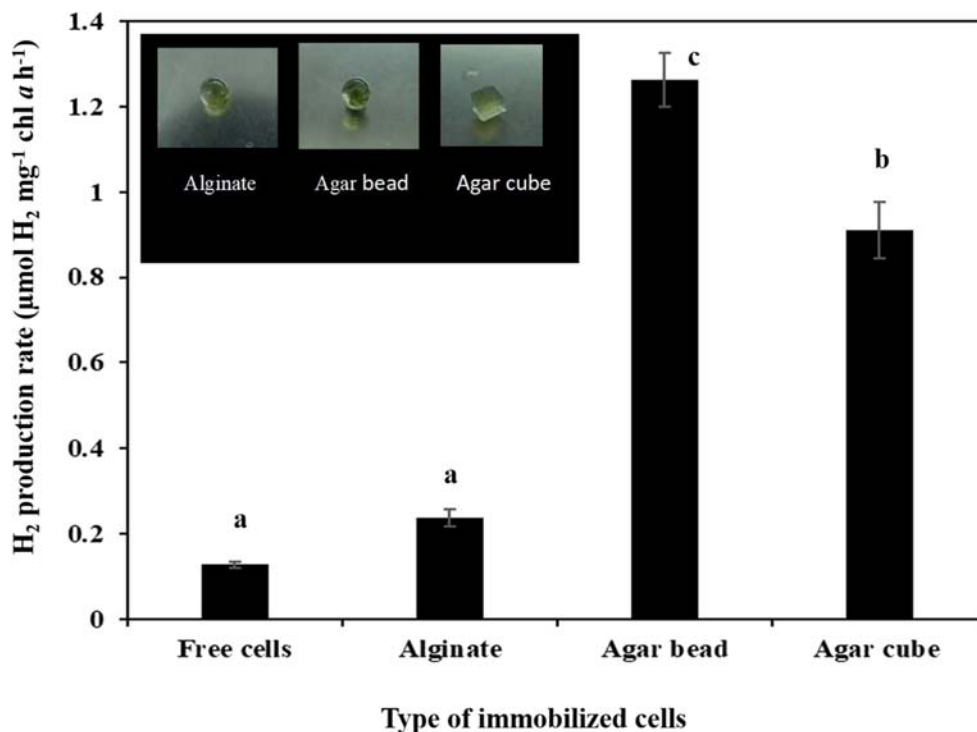
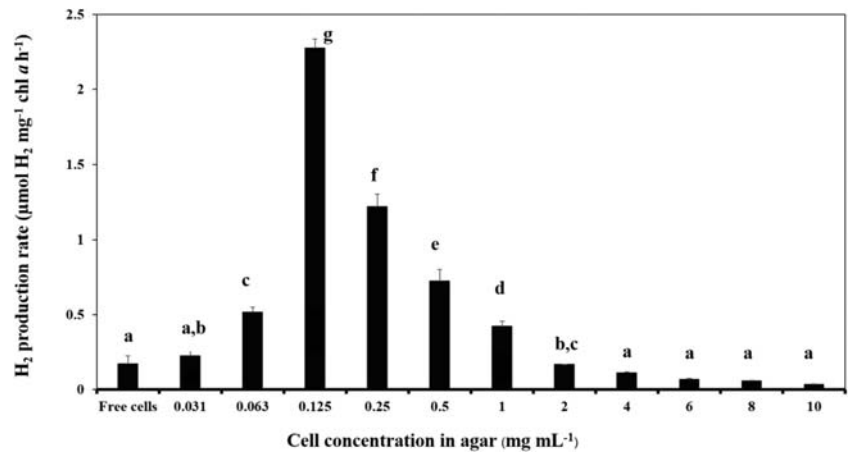


Fig. 5 Effect of cell concentration in agar bead on hydrogen production rate by immobilized cells of *Nostoc sp.* CU2561. Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters



in agar bead under optimized condition was $18.78 \pm 1.40 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$ or $629.13 \pm 1.51 \mu\text{mol H}_2 \text{ g}^{-1} \text{ dry weight h}^{-1}$. When comparing the hydrogen production efficiency of agar bead-immobilized *Nostoc sp.* CU2561 with those from different immobilized cells using various matrix supports, *Nostoc sp.* CU2561 had similar hydrogen production to that of agar cube-immobilized *Aphanothece halophytica* under the same hydrogen production condition of BG11-N, but had about a 9-fold higher hydrogen production under BG11-N-S condition when compared with agar cube-immobilized *Anabaena siamensis* TISTR 8012. When compared with *Lynghya perelegans* immobilized in agar cube and alginate bead under BG11, about a 4-fold higher hydrogen production was found in *Nostoc sp.* CU2561 immobilized in agar bead under BG11-N-S. Under BG11-N-S supplemented with fructose, *Nostoc sp.* CU2561 immobilized in

agar bead showed about a 2.3-fold higher hydrogen production than *Microcystis aeruginosa* immobilized in agar cube under MA medium supplemented with glucose. The recently reported hydrogen production of $17.30 \pm 0.24 \mu\text{mol H}_2 \text{ mg}^{-1} \text{ chl } a \text{ h}^{-1}$ by agar cube-immobilized *Fischerella muscicola* TISTR 8215 was slightly lower than that of *Nostoc sp.* CU2561 immobilized in agar bead under optimized condition in this study.

Sustaining H₂ production by agar bead-immobilized cells

Hydrogen production for 3 cycles by agar bead-immobilized cells of *Nostoc sp.* CU2561 under BG11-N (agar bead), BG11-N-S (agar cube), and BG11-N-S (agar bead) is shown in (Fig. 9). The results indicate that agar bead-immobilized cells could continuously produce hydrogen for 3 cycles. In the first cycle, hydrogen production was prolonged up to 120 h. Immobilized cells in agar beads incubated in BG11-N-S showed the highest hydrogen yield of $2.02 \pm 0.06 \mu\text{mol H}_2 \text{ mL}^{-1}$ compared with those of 1.12 ± 0.08 and $1.48 \pm 0.05 \mu\text{mol H}_2 \text{ mL}^{-1}$ incubated in BG11-N (agar bead) and BG11-N-S (agar cube), a 1.8-fold and a 1.36-fold increase, respectively. In the 2nd cycle, the hydrogen yield was decreased to about 60% of that in the first cycle. In the 3rd cycle, agar bead-immobilized cells almost lost their capacity to produce hydrogen, retaining about 10% yield of that obtained in the 2nd cycle.

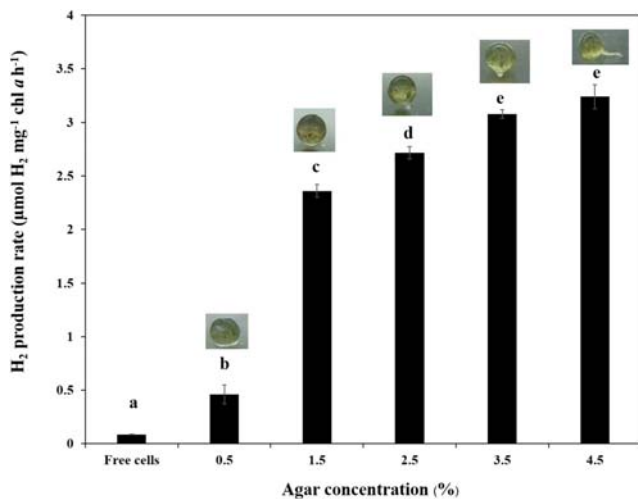


Fig. 6 Effect of agar beads concentration on hydrogen production rate by immobilized cells of *Nostoc sp.* CU2561 at cell concentration of 0.125 mg mL^{-1} . Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters. The shape of the bead at different agar bead concentrations is shown above the bar graph

Discussion

During sulfur deprivation, oxygenic photosynthesis is decreased because sulfur is an important component of D1 protein which is essential for photosystem II-mediated O₂ evolution (Melis et al. 2000). S deprivation induces a partial inactivation of PSII leading to anaerobiosis (Antal et al. 2003). Therefore, the decreased O₂ evolution resulted in an

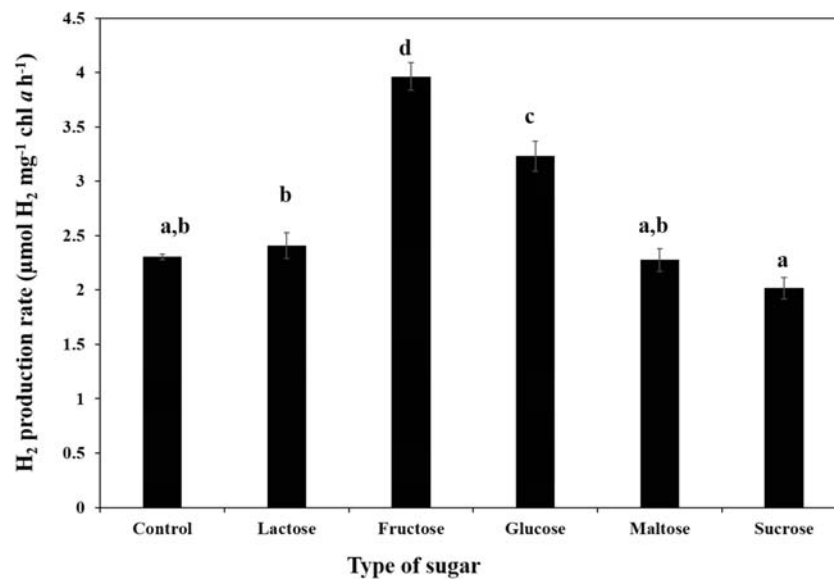


Fig. 7 Effect of sugar sources on hydrogen production rate by immobilized cells of *Nostoc* sp. CU2561. The 7-day BG11-N grown cells were transferred to BG11-N S medium supplemented with 0.5% (w/v) of various kinds of sugar and incubated under light for 24 h. Then, cells were

immobilized in 1.5% (w/v) agar, transferred to a glass vial before detecting H₂ production. Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters

enhancement of hydrogen production due to the decreased inhibition of O₂ on nitrogenase and hydrogenase activities.

The main purpose of the present study is to find suitable support material to immobilize cells to enhance the capacity of cells to produce hydrogen. The hydrogen production by *Nostoc* sp. CU2561 had the highest production rate when immobilized in 1.5% (w/v) agar bead followed by that in 1.5% (w/v) agar cube and in 4% alginate bead, whereas the free cells had the lowest hydrogen production rate (Fig. 4). Our results were in agreement with those of the previous study

in immobilized cells of cyanobacterium *Lyngbya perelegans*. The hydrogen production of *L. perelegans* immobilized in agar and alginate was 2–4-fold higher than that of free cells (Anjana and Kaushik 2014).

A previous study in the green alga *Chlorella* sp. showed that cell immobilization with agar can protect bidirectional hydrogenase from the outer environments including O₂ which is a strong hydrogenase inhibitor (Song et al. 2011). When comparing the different shapes of agar, according to the mathematical calculation, a bead has less

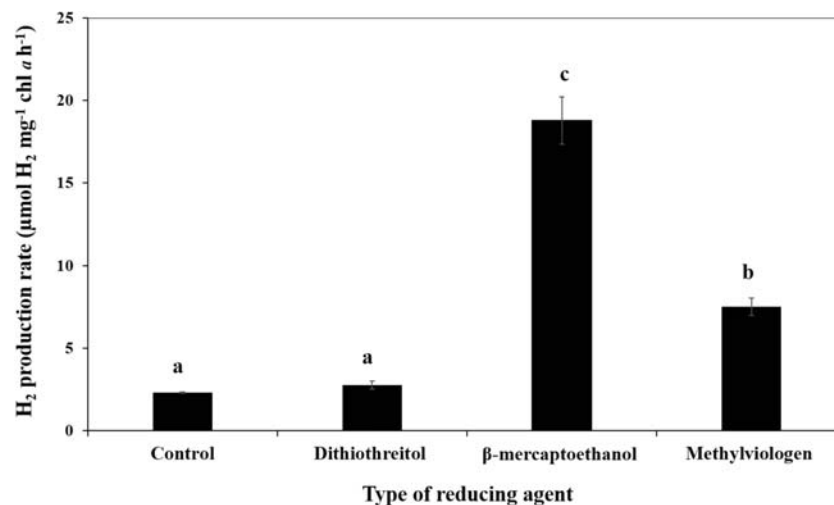


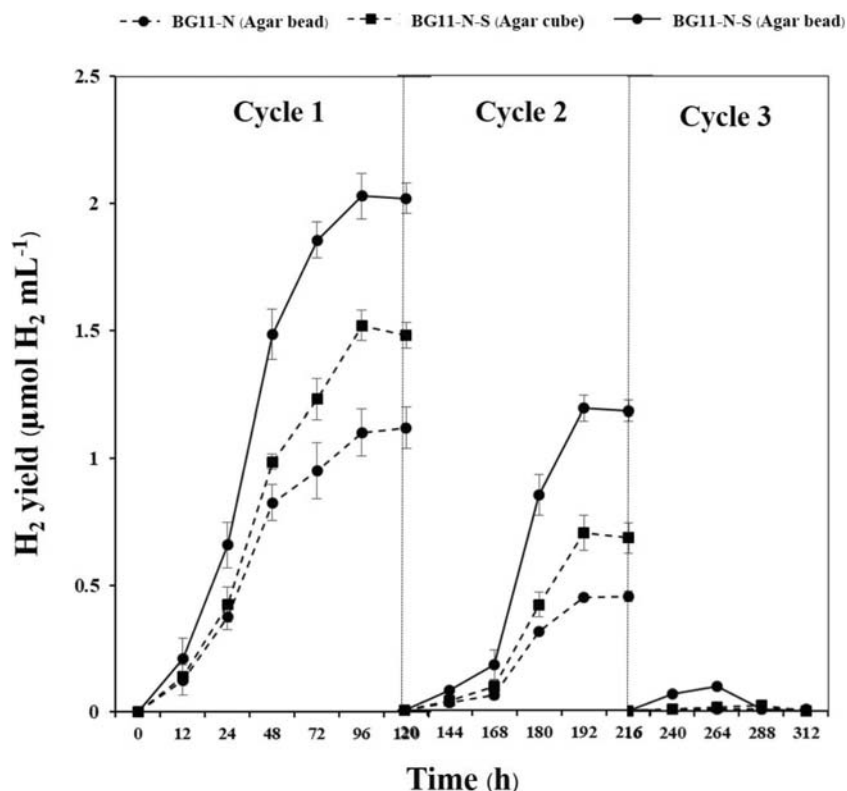
Fig. 8 Effect of reducing agent on hydrogen production rate by immobilized cells of *Nostoc* sp. CU2561. Cells were grown in BG11-N medium for 7 days. Cells were washed and transferred to BG11-N S medium supplemented with 5 mM of dithiothreitol, β-mercaptoethanol, and methyl viologen before immobilization in 1.5% (w/v) agar beads.

The immobilized cells were adapted under light for 24 h. before transferring to a glass vial for H₂ production determination. Data are means \pm standard deviations from three replicates. Significant differences (p value < 0.01) are indicated by different letters

Table 1 Comparison of the maximum hydrogen production from immobilized cells of *Nostoc* sp. CU2561 with other immobilized cyanobacterial strains

Species/strain	Media		Maximum H ₂ production rate			References
	Type of immobilized cells	Growth condition	H ₂ production condition	μmol H ₂ mg ⁻¹ chl <i>a</i> h ⁻¹	nmol h ⁻¹ mg ⁻¹ DW or μmol H ₂ g ⁻¹ mL h ⁻¹ L ⁻¹ dry weight h ⁻¹	
<i>Nostoc</i> sp. CU2561	Agar bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N) (N-deprived)	1.20 ± 0.08	40.88 ± 0.40	This study
	Agar bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived)	2.36 ± 0.06	80.94 ± 0.60	This study
	Agar bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived) with 5%(w/v) fructose	3.96 ± 0.13	127.92 ± 1.32	This study
	Agar bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived) with 5 mM β-mercaptoethanol	18.78 ± 1.40	629.13 ± 1.51	This study
	Agar cube	BG11 using salt solution	BG110 (-N) (N-deprived) with salt solution	-	39.14 ± 1.51	Pansook et al. (2019)
<i>Aphanothece halophytica</i> TISTR 8012	Agar cube	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived)	0.25 ± 0.03	-	Taikhao and Phunpruch (2017)
	Alginate bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived)	0.33 ± 0.02	-	Taikhao and Phunpruch (2017)
	Alginate bead	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N-S) (N-S-deprived) with 5%(w/v) fructose	0.68 ± 0.02	-	Taikhao and Phunpruch (2017)
<i>Lyngbya perelegans</i>	Agar cube	BG11	BG11	-	19.79 ± 0.71	Anjana and Kaushik (2014)
<i>Microcystis aeruginosa</i>	Agar cube	MA medium	MA medium (-S) (-S-deprived) with 30 mM glucose	-	21.49 ± 0.14	Anjana and Kaushik (2014)
<i>Fischerella muscicola</i> TISTR 8215	Agar cube	BG11 ₀ (-N) (N-deprived)	BG11 ₀ (-N) (N-deprived)	17.30 ± 0.24	-	Rashid et al. (2009)
						Wurthithien et al. (2019)

Fig. 9 Hydrogen yield of repeated reactor processes by immobilized cells of *Nostoc* sp. CU2561 at cell concentration of 0.125 mg mL^{-1} in 1.5% (w/v) agar prepared and incubated in BG11-N (agar bead), BG11-N-S (agar cube), and BG11-N-S (agar bead). Data are means \pm standard deviations from three replicates



surface area than a cube at the same volume; i.e., the surface area per unit volumes of bead and cube were 4.8 and 6.0, respectively. This suggested that the diffusion of O_2 in the outer environment into the agar bead was less than that into the agar cube, which might lead to lower inhibition of nitrogenase and hydrogenase in agar bead-immobilized cells. Another possibility for higher hydrogen production rate in agar bead-immobilized cells is that during the preparation of agar cube-immobilized cells, the immobilized cells suffered cell damage as a result of cutting into a cube shape. Furthermore, the spherical shape of agar beads can impart the mechanical and chemical stability to the beads (Lee et al. 2013). It was also reported that in alginate beads a nonspherical shape of the beads reduced gel bead strength compared with spherical beads (Hajry et al. 1999).

The highest hydrogen production rate was obtained when immobilization with $0.125 \text{ mg cell dry wt mL}^{-1}$ agar (Fig. 5). Previously, it was shown that immobilized cells of *Chlamydomonas reinhardtii* in alginate films had decreased hydrogen production rates when cell concentrations were increased (Kosourov and Seibert 2009). This might be because the mechanical stability of the alginate with immobilized cells was decreased when increasing the cell concentration (Antal et al. 2016). Too high cell density can cause a shading effect leading to a decline of light penetration reaching the cells which can affect hydrogen production. *Fischerella muscicola* TISTR 8215 in both free and agar-immobilized cells had a

decreased hydrogen production when too high cell concentration was used (Wutthithien et al. 2019). In addition, the cell density may affect the porosity of the agar matrix. The use of too high cell density can lead to an increased porosity of agar cubes resulting in easier diffusion of O_2 into the matrix (Anjana and Kaushik 2014).

The hydrogen production by agar bead-immobilized *Nostoc* sp. CU2561 showed an increase of hydrogen production rate with an increase of agar concentration (Fig. 6). A high degree of gel polymerization occurs upon increasing gel concentration resulting in a decrease of gel porosity. Thus, the decreased gel pore size can hinder the access of O_2 existing in external environment rendering both nitrogenase and hydrogenase more active. Previously, the filamentous cyanobacterium *Calothrix* had increased and prolonged hydrogen production when immobilizing cells with 4% (w/v) sodium alginate (Leino et al. 2012). On the other hand, agar bead concentration lower than 1.5% (w/v) significantly decreased hydrogen production (Fig. 6). Low agar concentration results in a low strength or less stable gel and this increases the possibility of the leakage of cells from the gel leading to a decreased hydrogen production. Therefore, the suitable concentration of support material for immobilization is important for the improvement of H_2 production by immobilized cells.

Cells of *Nostoc* sp. CU2561 immobilized in 1.5% (w/v) agar beads showed the highest hydrogen production rate when incubated in BG11-N-S medium containing 0.5% (w/v)

fructose (Fig. 7). It is worth mentioning that a 2-fold increase of hydrogen production was observed in *A. siamensis* TISTR 8012 immobilized in alginate bead when supplementing 0.5% (w/v) fructose to BG11-N-S medium (Taikhao and Phunpruch 2017). Cyanobacteria can use CO₂ as a source of carbon in photosynthesis. Moreover, they are also able to use various sugars for heterotrophic growth (Smith et al. 1987). The presence of some sugars strongly induces higher hydrogen production by *Nostoc* sp. CU2561, possibly due to the utilization of organic substrates as sources of energy (ATP) and reductants of nitrogenase (Neuer and Bothe 1985). Although we did not determine the utilization of different sugars by *Nostoc* sp. CU2561, there was a possibility that higher hydrogen production under fructose was partly ascribed to its better utilization than other sugars as previously reported in *Anabaena* sp. strain CH3 (Chen et al. 2008). Similarly, fructose uptake rate by *A. siamensis* strain TISTR 8012 was faster than other sugars during 12-h incubation (Khetkorn et al. 2010). This might partly explain the differences in hydrogen production by different sugars in cyanobacteria. Moreover, fructose at 40 mM (0.72%, w/v) was shown to increase heterocysts size and frequency of *Anabaena variabilis* (Lang et al. 1987), a condition that promotes nitrogen fixation with a subsequent increase in hydrogen production. The upregulation of *nifD* and to a less extent *hoxH* expression level was reported for *A. siamensis* TISTR 8012 in the presence of fructose (Khetkorn et al. 2010). Fructose has an important role in N₂ fixation in heterocyst because its presence resulted in increased respiration leading to a decreased O₂ level, a condition that supports nitrogenase activity (Haury and Spiller 1981).

The observed potential of fructose to increase H₂ production is interesting. In the future, we can utilize wastewater from different sources to supply fructose for an enhancement of hydrogen production by filamentous N₂ fixing cyanobacteria.

When considering the effect of reducing agents on H₂ production by agar bead-immobilized *Nostoc* sp. CU2561, the highest hydrogen production rate was obtained under incubation with BG11-N-S medium containing 5 mM β-mercaptoethanol (Fig. 8). The supplementation of β-mercaptoethanol to enhance hydrogen production by 1.5- and 2-fold was also reported in *Arthrospira* sp. PCC 8005 (Raksajit et al. 2012) and *Tetraspora* sp. CU2551 (Maneeruttanarungroj et al. 2010), respectively. The presence of reductant in the medium enabled cyanobacterial cells to generate energy and excess electrons which could increase H₂ production. Enhancement of hydrogenase activity was observed in *Synechocystis* sp. PCC 6803 grown in BG11-N-S medium containing 0.75 mM β-mercaptoethanol (Baebprasert et al. 2010). Although β-mercaptoethanol is a cytotoxic substance, no discernible difference in cell growth was detected when incubating *Nostoc* sp. CU2561 cells in BG11 medium

with and without 5 mM β-mercaptoethanol for 24 h (Fig. S2). Methyl viologen also increased hydrogen production in immobilized *Nostoc* sp. CU2561, but to a lower extent than β-mercaptoethanol (Fig. 8). It is possible that the stimulatory effect of methyl viologen on hydrogen production might be due to its stability impact on the hydrogenase enzyme as previously reported in *Escherichia coli* (Adams and Hall 1979).

Another possible role of β-mercaptoethanol in increasing hydrogen production could be ascribed to their O₂ removal activity creating more anaerobiosis. Previously, *Chlamydomonas gloeopara* and *Scenedesmus obliquus* with enhanced hydrogen production showed improved photosynthetic O₂ consumption by treatment with chemical reducing agents (Márquez-Reyes et al. 2015). In this regard, it is worth mentioning that the use of chemical O₂ scavenging system has been recently shown to efficiently improve hydrogen production in *C. reinhardtii* (Khosravitarbar and Hippler 2019).

Very recently, a highly efficient hydrogen production was reported for agar cube-immobilized *Fischerella muscicola* TISTR 8215 with about a 4.4-fold increased production compared with the suspended cells (Wutthithien et al. 2019). Although *Nostoc* sp. CU2561 in this study had a low hydrogen production efficiency compared with that in *F. muscicola* TISTR 8215, upon immobilization using agar bead, its hydrogen production was increased by about 9-fold (Fig. 4). This suggested that immobilization of filamentous cyanobacterial cells in agar bead was likely to result in more improvement of hydrogen production efficiency. The immobilization of filamentous cyanobacterium *Nostoc* sp. CU2561 in agar beads is safe, cheap, easy to harvest, and simple to scale up at industrial level in the future.

Another advantageous aspect of agar bead-immobilized cells in this study is due to the sustained production of hydrogen. The repeated 3-cycle hydrogen production by agar bead-immobilized cells of *Nostoc* sp. CU2561 under BG11-N-S (Agar bead) showed a total accumulated hydrogen yield of $3.35 \pm 0.09 \mu\text{mol H}_2 \text{ mL}^{-1}$ (Fig. 9). In this regard, agar bead-immobilized *Nostoc* sp. CU2561 had about a 2-fold lower hydrogen yield than that of agar cube-immobilized *F. muscicola* TISTR 8215 with a total accumulated hydrogen yield of $7.8 \mu\text{mol H}_2 \text{ mL}^{-1}$ (Wutthithien et al. 2019). However, it should be noted that the suspended cells of *F. muscicola* TISTR 8215 had a 20-fold higher hydrogen production than the suspended cells of *Nostoc* sp. CU2561. Thus, the use of agar beads for the immobilization of *Nostoc* sp. CU2561 facilitated the improvement of its hydrogen production, whereas less improvement was observed in *F. muscicola* TISTR 8215 immobilized in agar cube. Moreover, agar bead might be slightly better than agar cube, in terms of hydrogen production prolongation, where *Nostoc* sp. CU2561 could prolong hydrogen production up to 120 h compared with 108 h in *F. muscicola* TISTR 8215.

In conclusion, we isolated *Nostoc* sp. CU2561 with an ability of enhanced hydrogen production when immobilization with agar beads. The results showed the potential of a new immobilization strategy using agar bead immobilization for hydrogen production. The hydrogen production rate by *Nostoc* sp. CU2561 was increased when the cells were grown in BG11-N medium. In addition, the hydrogen production rate was further increased in cells incubated under nitrogen and sulfur deprivation. Moreover, the highest hydrogen production rate by agar bead-immobilized cells was observed with a cell concentration of 0.125 mg dry wt mL⁻¹ agar bead and a final agar concentration of 1.5% (w/v) agar bead. The supplementation of 0.5% (w/v) fructose increased hydrogen production rate, an approximately 1.7-fold higher than that incubated in the sugar-free BG11-N-S medium in immobilized cells. Moreover, the reducing agent β-mercaptoethanol markedly increased hydrogen production about 8.2-fold in immobilized cells. In addition, agar bead-immobilized cells could continuously produce hydrogen for 3 cycles where the production by immobilized cells was prolonged up to 120 h during the first cycle.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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