Journal of Biotechnology: X 4 (2019) 100016

Contents lists available at ScienceDirect

Journal of Biotechnology: X

journal homepage: www.journals.elsevier.com/journal-of-biotechnology-x

Immobilized heterocysts as microbial factories for sustainable nitrogen fixation

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ARTICLE INFO

Keywords:
N_2-fixation
Cyanobacteria
Immobilization
Nitrogenase
Thin-films
Heterocysts

ABSTRACT

A novel thin-layer biocatalyst for photosynthetic N_2 fixation and H_2 photoproduction was assembled using a Ca^{2+}-alginate matrix and heterocysts isolated from wild-type *Anabaena* sp. PCC 7120 filaments. Compared to suspension heterocysts, heterocysts entrapped in Ca^{2+}-alginate films showed improved stability of the nitrogenase system. While suspension heterocysts lost nitrogenase activity within 24 h, immobilized heterocysts supported nitrogenase activity for up to 125 h. The maximum specific rate of acetylene reduction was the same in both cases (~0.4 μmol C_2H_2 mg Chl^{-1} h^{-1}), but the catalyst with entrapped heterocysts required a much longer time to achieve the maximum rate (60 h instead of 3 h in suspension). Simultaneously with acetylene reduction, the immobilized heterocysts were able to photoproduce H_2 for 125 h, yielding up to 1.1 mmol H_2 mg Chl^{-1}. The absence of acetylene increased the H_2 photoproduction rate to a maximum of 25–30 μmol H_2 mg Chl^{-1} h^{-1}, and the catalyst was capable of H_2 photoproduction for 190 h, yielding up to 2.5 mmol H_2 mg Chl^{-1}. The recovery of the catalyst with entrapped heterocysts was achieved through placing the cells in a N_2 atmosphere for 24 h. This engaged a second cycle of H_2 photoproduction, which lasted for another 240 h (10 days), thus yielding ~3 mmol H_2 mg Chl^{-1} in total after 454 h. Together, these findings demonstrate great potential for a heterocyst-based thin-layer platform for the sustainable production of chemicals and biofuels.

1. Introduction

Photosynthetic organisms use sunlight as an energy source and raw materials (CO_2, water and some minerals) for the synthesis of energy-rich organic compounds. The release of O_2 is a by-product of water oxidation. Photosynthetic cyanobacteria and algae are considered to be third generation sustainable feedstocks for blue biorefineries and the associated chemicals industry (Laurens et al., 2017). Moreover, they hold great potential as microbial cell factories for employment in the sustainable production of various targeted fuels and chemicals (Lips et al., 2018; Khan et al., 2018).

N_2-fixing cyanobacteria are able to employ photosynthetically generated ATP as an energy source to break the strong triple bond between atoms of atmospheric nitrogen (N_2) to produce ammonia. This reaction is catalyzed by the nitrogenase enzyme complex. The most studied and well characterized of these enzymes is the multimeric molybdenum (Mo) - containing nitrogenase. It has two components: (i) dinitrogenase reductase, an iron protein (Fe-protein) of 32–40 kDa; and (ii) dinitrogenase, a tetrameric iron-molybdenum protein (MoFe-protein) of 230–250 kDa. The dinitrogenase reductase transfers electrons from reduced ferredoxin (or flavodoxin) to the dinitrogenase protein, which in turn binds and reduces N_2 (Bothe et al., 2010). Besides conventional Mo-nitrogenase, some cyanobacteria possess a non-conventional Mo-nitrogenase (*e.g.* expressed in vegetative cells of *Anabaena variabilis* ATCC 29413 under anoxic conditions) or an alternative vanadium (V)-nitrogenase (Masukawa et al., 2009; Thiel and Pratte, 2014). The N_2-fixation process is always accompanied by the release of molecular hydrogen (H_2) as a byproduct. In the absence of N_2, nitrogenase catalyzes the reduction of protons to H_2, which stimulates the H_2 production yield in cyanobacterial cultures. Thus, N_2-fixing cyanobacteria hold particular potential for service in the sustainable production of hydrogen gas (Bandyopadhyay et al., 2010; Magnuson, 2019).

Nitrogenase is O_2 sensitive, with cyanobacteria having developed a number of strategies to protect its function. In the absence of combined nitrogen, many filamentous cyanobacteria undergo cell differentiation. In this process, unique specialized cells, known as heterocysts, differentiate from vegetative cells. Vegetative cells perform normal

Abbreviations: C_2H_2, acetylene; Fe-protein, iron protein; H_2, hydrogen; MoFe-protein, iron-molybdenum protein; N_2, nitrogen

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https://doi.org/10.1016/j.btecx.2020.100016
Received 23 November 2019; Received in revised form 14 February 2020; Accepted 21 February 2020
Available online 25 February 2020

photosynthesis including water-oxidation and O_2 evolution at Photosystem II, production of NADPH and ATP, and CO_2 fixation. In contrast, heterocysts are specialized primarily in N_2 fixation and do not perform CO_2 fixation. Heterocysts are spaced at regular intervals among vegetative (photosynthetic) cells and provide a microoxic environment suitable for the enzymes involved in N_2-fixation (Haselkorn, 1978; Wolk, 2000; Flores and Herrero, 2010; Muro-Pastor ans Hess, 2010). The microoxic environment inside heterocysts is maintained through the employment of a few key mechanisms: (i) an elevated rate of O_2 consumption enabled via the activity of respiratory terminal oxidases (Wolk et al., 1994; Bothe et al., 2010), and the heterocyst specific flavodiiron protein (Ermakova et al., 2014); (ii) a lack of active O_2-evolving Photosystem II complex; and (iii) the development of a thick multilayered cell wall which acts as a barrier to gas exchange (Bothe et al., 2010), with gas exchange mainly occurring via terminal pores connecting vegetative cells and heterocysts (Walsby, 2007). Vegetative cells and heterocysts are symbiotically associated, whereby vegetative cells perform photosynthetic CO_2 fixation and provide heterocysts with organic carbon intermediates, like sucrose (Lopez-Igual et al., 2012), and heterocysts provide vegetative cells with the fixed nitrogen required for cell growth. Importantly, the presence of Cytochrome *b6f* and Photosystem I in heterocysts provides light-induced stimulation of N_2 fixation (Magnuson, 2019).

The cyanobacterial N_2-fixation process contrasts the current, yet century-old, industrial production of ammonia by the Haber-Bosch process, which is extremely demanding in respect to both energy (high temperature and pressure) and resources (purified N_2 and H_2). In order to sustain the high global demand for ammonia fertilizer required to feed a rapidly increasing population and to minimize the adverse environmental effects of production, a shift to modern and efficient green processes are required. In this respect, photosynthetic cell factories (Grizeau et al., 2015) and the ammonia artificial leaf concept (Martin et al., 2018) are promising renewable approaches. Ammonium excretion by N_2-fixing cyanobacteria is a natural process, which occurs in cyanobacteria-plant symbiosis. The excretion process can also be induced by blocking ammonium assimilation, using glutamine synthetase inhibitors (Musgrave et al., 1982; Brouers and Hall, 1986), or through genetic manipulation (Zhang et al., 2007; Bui et al., 2014). Whilst research on N_2 fixation using isolated heterocysts has been undertaken as early as the 1970s, only short production periods of 20–30 min were investigated (Peterson and Wolk, 1978; Lockau et al., 1978). Thus, it may have been generally accepted that activity of isolated heterocysts could not be preserved for the extended time periods required for a feasible industrial process.

The immobilization of microorganisms and biomolecules within polymer matrices is one approach to extending the lifetime of microbial and/or cell based production systems (Pressi et al., 2003; Nieto et al., 2005; Desimone et al., 2006; Gautier et al., 2006; Dickson et al., 2009). Indeed, improved stability and H_2 photoproduction activity have been demonstrated in photosynthetic cyanobacteria and microalgae immobilized in thin-films compared to suspension cultures, with months-long production periods being maintained (Leino et al., 2012; Kosourov and Seibert, 2009; Kosourov et al., 2017). Immobilization is not limited to thin films, with cubes (Wutthithien et al., 2019) and hydrogel lenses (Homburg et al., 2019) more recent approaches. Alginate beads have been a popular approach to immobilization for several decades and have been employed for a variety of applications, including nitrogen fixation. Whole cyanobacterial filaments immobilized in alginate beads demonstrated nitrogenase activity (measured as acetylene reduction) for 22 days (Musgrave et al., 1982), whereas H_2 photoproduction in thin-layer alginate films (a measure of nitrogenase activity) continued over 38 days (Leino et al., 2012).

Thus, in order to test a system suitable for future efficient and sustained production of ammonia and hydrogen gas, we have combined the two aforementioned approaches of heterocyst isolation and immobilization, and demonstrated a proof of concept for the thin-layer immobilization of cyanobacterial heterocysts for efficient solar-driven N_2 fixation. By fixing atmospheric nitrogen, engineered heterocyst microfilms will act as long-lived biocatalysts producing: (i) ammonia, which is an important bio-industrial compound, and can be used as a fertilizer or fuel; or (ii) H_2, which is an environmentally friendly, clean energy source.

2. Material and methods

2.1. Strains and growth conditions

The pre-experimental culture of wild type *Anabaena* sp. PCC 7120 was grown under diazotrophic conditions in $BG11_0$ medium (buffered with 10 mM TES–KOH, pH 8.2) without combined nitrogen. The cells were cultivated in 150 ml Erlenmeyer flasks containing 50 ml of $BG11_0$ medium by agitation on a shaker (120 rpm) at 30 °C with two-sided illumination of 70 µmol photons $m^{-2}s^{-1}$ photosynthetic active radiation (PAR) in the presence of air enriched with 3 % CO_2 (vol/vol).

The experimental cultures were grown for 7 days under the same conditions in 1 L Erlenmeyer flasks containing 500 ml of $BG11_0$. After reaching a chlorophyll (Chl) concentration of about 15 µg Chl ml^{-1}, (OD = 1) the cells were harvested by centrifugation for the heterocyst isolation procedure.

2.2. Isolation of heterocysts

Heterocysts were isolated under anoxic conditions as described in Razquin et al (2002) or Ermakova and Allahverdiyeva (2015), with some modifications. Harvested filaments were washed twice with buffer A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM $MgCl_2$) at 5000 g for 5 min. The biomass pellet was resuspended in 20 ml of buffer A containing lysozyme (1 mg ml^{-1}) in a 50 ml falcon tube closed with a gas-tight Suba-Seal®rubber septum (Sigma-Aldrich, USA), put in an ice bucket and flushed with argon for 25 min under room light. Then, the lysozyme-treated mixture was incubated at 37 °C for 1 h with continuous mixing on a shaker. Further experiments were performed under two different anaerobic conditions: (1) strict anoxic conditions using a vinyl anaerobic airlock chamber (Coy laboratory products, Michigan USA) or (2) local anoxic conditions using gas-tight vials sealed with rubber stoppers and Ar-flushed gas-tight syringes for heterocyst washing and sampling. Centrifugation was performed at room temperature, but cold buffer was used and heterocysts were kept on ice when possible. After lysozyme treatment, the filaments were washed twice with 20 ml anoxic buffer B (50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$) by centrifuging cells at 5000 g for 3 min. The cells were sonicated in ultrasonic cleaning baths for 5 min at 4 °C. Digested vegetative cells were removed by washing the cell mixture three times in 15–20 ml Buffer C (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM Na_2HPO_4, 5 mM KH_2PO_4) at 200–250 g for 3 min. The final pellet was resuspended in ~5 ml of buffer C. The quality of the heterocyst pellet was monitored under the light microscope. The isolation was considered successful if the content of vegetative cells fragments was below 1 %. After that, heterocysts were centrifuged once at 4,000 g for 3 min to remove the buffer from the pellet for immobilization. Heterocysts were used immediately after isolation. Usually, ~1.5 g of fresh weight heterocyst biomass was obtained from 1 l of culture.

2.3. Immobilization of heterocysts

The immobilization procedure was performed as described in Leino et al. (2012) with some modifications. Different to the original protocol, the Scotch-type tape was not applied to the template. All steps were performed under anaerobic conditions. Heterocyst pellets were resuspended in buffer C and mixed thoroughly with sterile 2 % Na-alginate (Sigma-Aldrich), using the formulation ratio: 0.6–3 g wet heterocyst weight, 0.5 ml buffer C and 1 ml 2 % alginate. After

immobilization, the Ca^{2+}-alginate thin-films with entrapped heterocysts were cut into 4 × 1 cm strips. The thickness of the films was determined by plastic mesh, used as a mechanical support, at around 100 µm. The total amount of Chl *a* was ~20–100 µg per 4 cm^2 film. Chl *a* values specific to each assay are detailed in the relevant figure legends.

2.4. Nitrogenase activity assay

Nitrogenase activity was determined by an acetylene reduction assay (Dilworth, 1966) performed on isolated heterocysts and immobilized heterocysts. The suspension heterocysts (4 ml) were transferred into 10 ml anoxic vials. The 4 cm^2 Ca^{2+}-alginate strips with entrapped heterocysts were transferred into 36 ml anoxic vials (1 strip per vial) containing 4 ml of buffer C supplemented with 20 or 200 mM sucrose for submerged cultivation. Volumes used were chosen to optimize the balance between sensitivity and accuracy of gas measurements. All vials were supplemented with 10 % acetylene and immediately placed in a growth chamber at 30 °C under continuous two sided-illumination at an intensity of 70 µmol photons $m^{-2}s^{-1}$. To prevent sedimentation, the vials with heterocyst suspensions were placed on a shaker operating at 100 rpm. In contrast, the vials with alginate strips were not mixed. Alginate strips were not observed to degrade over the time course of the experiment. Acetylene reduction activity was calculated on the basis of the chlorophyll (Chl) *a* content.

The ethylene content in the headspace of the vials was determined using a GC (Clarus® 580, PerkinElmer, Inc.) equipped with a Carboxen 1010 PLOT 30 m x 0.53 mm capillary column and a flame ionization detector (FID). Argon was used as a carrier gas. Due to the presence of the methanizer catalyst in the FID line, which is sensitive to hydrocarbons, the temperature of the FID detector was decreased to 150 °C and all samples were diluted 50–100 times. For this, 100 or 50 µl gas samples were taken with a gas-tight 50 µl Hamilton syringe (Hamilton Company, USA) from the headspace of the incubation vials containing heterocyst or *Anabaena* filaments and injected into oxic 5 ml vials. Then, 40 µl samples from 5 ml vials were injected into the GC. Calibration was performed with different concentrations of ethylene from 0.001 to 0.1 % (AGA, Finland) using the same dilution approach. All ethylene yields were corrected for its solubility in the medium and the partial pressure in the headspace of the vials. In all experimental setups, the level of acetylene was in excess throughout the experiment, determined by simultaneous monitoring of the acetylene peak.

2.5. Determination of H_2 gas

The amount of H_2 gas produced by immobilized heterocysts was monitored once a day using a GC (Clarus® 500, PerkinElmer, Inc.) equipped with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh). 150 or 75 µl of gas sample from the headspace of 36 ml vials was taken using a gas-tight 250 µl Hamilton syringe and injected into the GC. Argon was used as a carrier gas and calibration was performed with 0.5 % hydrogen in argon (AGA, Finland). The incubation condition of immobilized heterocysts was described in the nitrogenase activity assay section.

2.6. Chlorophyll determination

The Chl *a* contents of *Anabaena* filaments and non-immobilized heterocysts were determined by extraction in 90 % methanol after 15 min of heating at 65 °C. For extraction of Chl *a* from immobilized heterocysts, the thin-films were placed in 90 % methanol at +4 °C in darkness for 24 h. The Chl content was determined spectrophotometrically at 665 nm, using the equation Chl *a* (µg ml^{-1}) = 12.7 × $Abs_{665\ nm}$ (Lichtenthaler, 1987). All samples were centrifuged at 3000 g before measurements.

3. Results and discussion

3.1. Effect of O_2 level on nitrogenase activity of isolated heterocysts

It has previously been shown that the function of O_2-sensitive nitrogenase is protected by rapid scavenging via the heterocyst specific flavodiiron protein (Ermakova et al., 2014) or terminal oxidases (Bothe et al., 2010). However, considering that gas exchange occurs mainly via terminal pores connecting vegetative cells and heterocysts (Walsby, 2007), the destruction of the vegetative cell/heterocyst connection during heterocyst isolation under a normal air atmosphere could greatly enhance the O_2 partial pressure inside of heterocysts, and so also inhibit nitrogenase activity. Two different isolation conditions were thus explored in seeking a balance between a simple preparation suitable for industrial use, and a strictly limited O_2 exposure, to protect nitrogenase. These conditions were: (i) preparation under a local anoxic condition, whereby the gas phase contained less than 3 % O_2, and (ii) strictly limited O_2 exposure through the use of an anaerobic chamber, where O_2 levels were able to be kept at 0.1–0.3 %. It is known that, besides N_2, nitrogenase can catalyze the reduction of different substrates with a triple bond, including acetylene (Bothe et al., 2010). Therefore, the nitrogenase activity of isolated heterocyst suspensions is routinely measured as acetylene reduction. Application of the acetylene reduction assay demonstrated that both heterocyst isolation procedures produce samples with acetylene reduction profiles, which peaked quickly and then declined over time. Heterocysts isolated under the local anoxic conditions (Fig. 1A, open circles) demonstrated a maximum acetylene reduction rate of 0.4 µmol mg Chl^{-1} h^{-1}, observed just 30 min after the activity assay was started by the injection of 10 % acetylene. This rate is in the range of previously published maximum rates of 0.3–0.4 µmol mg Chl^{-1} $h^{-1}$for isolated heterocysts in suspension culture (see for example, Petersonand Wolk 1978; Lockau et al., 1978). Over the next 30 min, the rate of acetylene reduction decreased two-fold (Fig. 1A, 1 h, open circle), with only residual activity (0.05 µmol mg Chl^{-1} h^{-1}) observed after 3 h of incubation.

The heterocysts isolated under the anaerobic chamber condition also demonstrated a maximum acetylene reduction rate of approximately 0.4 µmol mg Chl^{-1} h^{-1}. However, this was achieved 3 h after the beginning of the experiment (Fig. 1A, 3 h, closed circle) and activity was deemed insignificant at the 24 h point. Thus, it appeared that the lower oxygen exposure did not increase the maximum rate of acetylene reduction, but did delay and extend the active phase of the enzyme, resulting in prolonged nitrogenase activity.

It has previously been shown that the isolation of heterocysts in an atmosphere containing H_2 strongly enhances nitrogenase activity (Peterson and Wolk, 1978; Kumar et al., 1982), with recent work demonstrating that H_2 is a prerequisite for the binding of N_2 to the active site of the nitrogenase enzyme (Khadka et al., 2017). Thus, we investigated the effect of 10 % H_2 on heterocysts, which were isolated in the presence of 3 % O_2 and had lost almost all activity by 3 h of incubation (Fig. 1B). After flushing the same suspension of heterocysts with 10 % H_2 for 10 min, we applied another acetylene reduction assay test. In this test, the acetylene reduction rate responded as in the initial experiment, with an increase from 0.05 µmol mg Chl^{-1} h^{-1} (t = 0 min, white column Fig. 1B) to 0.47 µmol mg Chl^{-1} h^{-1} (t = 40 min of incubation, grey column), with the rate again dropping by the t = 60 min point (0.15 µmol mg Chl^{-1} h^{-1}). This response demonstrated the ability to regenerate the heterocyst suspension for nitrogenase activity by the introduction of H_2.

3.2. Nitrogenase activity of immobilized heterocysts: acetylene reduction

It has been shown that the immobilization of cyanobacterial filaments in polymer beads allows for sustained periods of nitrogen fixation (Musgrave et al., 1982) and increases the rates of acetylene reduction compared to filaments in suspension. This is due to increased

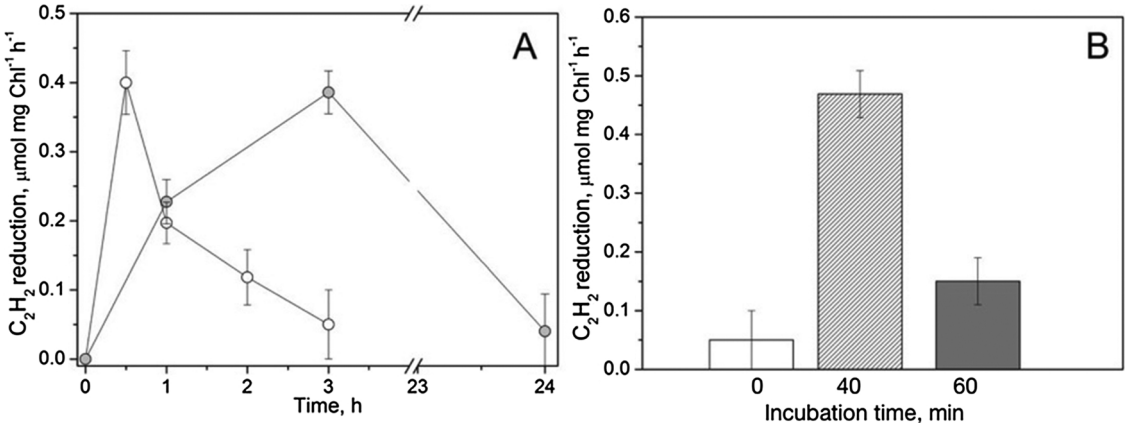

Fig. 1. Nitrogenase activity of heterocyst suspensions measured as acetylene reduction rates. (A) Heterocysts isolated under local anoxic conditions (open circle) and in a strictly anaerobic chamber (grey circle). (B) The effect of H_2 on the nitrogenase activity of heterocysts isolated under the local anoxic condition and subjected to the acetylene reduction assay for 3 h. The acetylene reduction activity was measured before flushing with 10 % H_2 (t = 0 min, white column); and after 10 min flushing with 10 % H_2 followed by 40 min (t = 40 min, grey column) and 60 min (t = 60 min, dark grey column) incubation in the atmosphere of 10 % acetylene in argon. Heterocysts were isolated from *Anabaena* sp. PCC7120 cells (see materials and methods for more details) using isolation media containing 200 mM sucrose. For the nitrogenase activity assay, the samples were incubated in an atmosphere of 10 % acetylene in argon, with continuous shaking (100 rpm) and 70 µmol photons $m^{-2}s^{-1}$ illumination at 30 °C (3 biological repetitions +/− SD).

photosynthetic electron transport and stabilized enzyme activities (Brouers and Hall, 1986). In an attempt to prolong the nitrogenase activity of isolated heterocysts, we immobilized heterocyst cells in thin-layer alginate films. Due to the positive influence of strict anaerobic isolation on prolonged heterocyst activity, the isolation of heterocysts and their entrapment in alginate films was performed in an anaerobic chamber.

Where reductants have been supplied in heterocyst isolation, a good balance between osmotic protection and inhibition of nitrogenase has been demonstrated for isolation in suspension culture at 200 mM sucrose (Jensen et al., 1986). In order to determine the effects of exogenous reductant on the nitrogenase activity of immobilized heterocysts, we undertook experiments on the immobilized heterocyst cells in buffer containing either 20 mM or 200 mM sucrose.

The profiles of the immobilized heterocysts clearly demonstrated further improvements in prolonged nitrogenase activity compared to suspension based heterocysts, with acetylene reduction activity detectable for at least 3 times longer. Rather than high initial rates of activity, immobilized heterocysts reached maximum acetylene reduction rates at around 60 h, after which steady declines were observed (Fig. 2A). The immobilized heterocysts incubated in buffer containing 200 mM sucrose showed higher acetylene reduction rates than those in 20 mM sucrose (maximum rates being 0.36 and 0.16 µmol mg Chl^{-1} h^{-1}, respectively). However, the rates did not exceed those of isolated suspension heterocysts in 200 mM sucrose buffer (~ 0.4 µmol mg Chl^{-1} h^{-1}, Fig. 1A).

It has been previously suggested that Mo-nitrogenase allocates all electrons to the reduction of acetylene, whereas V- or Fe-nitrogenases can produce a significant amount of H_2 as a byproduct during acetylene reduction assays (Bothe et al., 2010). Surprisingly, the concentration of H_2 determinedat the conclusion of the acetylene reduction assay (125 h) demonstrated that immobilized *Anabaena* heterocysts with Mo-nitrogenase were able to produce H_2 during the time that they performed C_2H_2 reduction (Fig. 2B). The addition of 200 mM sucrose was beneficial to H_2 production, with an approximately 2-fold increase compared to 20 mM sucrose (1.07 ± 0.2 vs 0.68 ± 0.1 mmol H_2 mg Chl^{-1}, respectively). Nevertheless, the conversion efficiency of sucrose to H_2 was higher in 20 mM samples (0.7 *vs* 0.4 mol H_2 produced /mol sucrose consumed in 20 mM and 200 mM samples, Table 1), indicating the absence of significant limitation for diffusion of sucrose through the

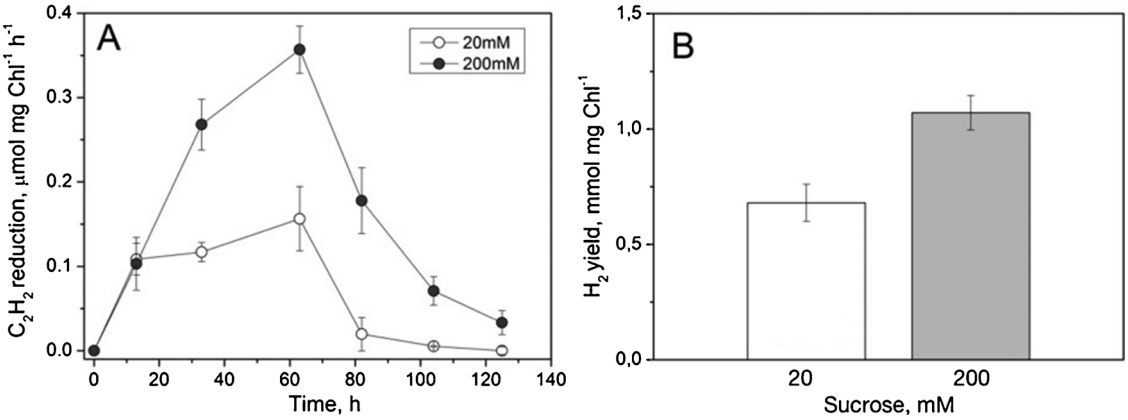

Fig. 2. Nitrogenase activity of immobilized heterocysts. (A) Acetylene reduction rate of immobilized cells supplemented with 20 mM (open symbols) or 200 mM (closed symbols) sucrose. The isolated heterocysts were immobilized in thin-alginate films (see material and methods for more details) and supplemented with 20 or 200 mM sucrose. (B) The final H_2 production yields observed at the conclusion of the acetylene reduction assay. Heterocysts were isolated as described in the methods section and placed in medium containing 20 or 200 mM sucrose. Samples were incubated in an atmosphere of 10 % acetylene in argon, without shaking and 70 µmol photons $m^{-2}s^{-1}$ illumination at 30 °C. H_2 yield was measured from the vials incubated in the acetylene reduction assay for 125 h. The Chl concentration was about 70 µg per strip.

Table 1
The amount of sucrose utilized by heterocysts of *Anabaena* PCC 7120 under different experimental conditions.

Initial concentration of sucrose, mM	Utilized sucrose, mmol mg Chl^{-1}	
	Immobilized heterocyst (acetylene reduction at 125 h)	Immobilized heterocyst (H_2 production at 168 h)
20	0.93 ± 0.04	0.99 ± 0.2
200	2.57 ± 0.45	3.38 ± 0.68

alginate matrix.

The results obtained demonstrate that the immobilization of heterocysts in a thin-layer alginate film increased the lifetime of heterocysts from a few hours (Fig. 1, suspension) to five days (Fig. 2), under our experimental conditions.

3.3. Nitrogenase activity of immobilized heterocysts: hydrogen photoproduction

Following the detection of H_2 gas produced by immobilized heterocyst cells during acetylene reduction assays, we performed a more detailed study of immobilized heterocyst H_2 photoproduction capacity under standard H_2 production conditions (only argon gas in the headspace). In this assay, heterocysts were again supplemented with either 20 or 200 mM sucrose.

Similar to observations of the acetylene reduction assay (Fig. 2B), H_2 photoproduction of heterocysts supplemented with 200 mM sucrose was higher than those supplemented with 20 mM (Fig. 3A). Once the heterocysts began producing H_2 at 24 h, the photoproduction rates of the 200 mM supplemented heterocysts were also higher over the course of the experiment. For both 20 and 200 mM sucrose supplemented heterocysts, production rates demonstrated a period of higher activity which ranged 24–72 h before declining afterwards (Fig. 3B). At 168 h, the cells supplemented with 20 mM sucrose stopped H_2 evolution, whereas the cells supplemented with 200 mM sucrose were still producing H_2 at a rate of ~9.21 μmol H_2 mg Chl^{-1} h^{-1}. Nevertheless, the conversion efficiency of sucrose to H_2 was ca. two times higher in 20 mM sucrose than in 200 mM (2.0 *vs* 0.8 mol H_2 /mol sucrose consumed, Table 1).

Next, we tested if flushing the immobilized heterocysts with lowered activity (173 h) with N_2 (a substrate) would increase nitrogenase activity. The cells incubated 24 h in a N_2 atmosphere continued to produce H_2 in an Ar atmosphere for the next 10 days (Fig. 4A) at a rate of about 5 μmol H_2 mg Chl^{-1} h^{-1} (Fig. 4B).

In order to find an optimal concentration of heterocyst cells to employ in the thin-layer films, we evaluated the effect of total Chl (related to the amount of heterocysts) on H_2 photoproduction. Heterocysts immobilized at a total Chl of 40 μg demonstrated the maximum specific H_2 production rates, averaging 25 μmol mg Chl^{-1} h^{-1} (Table 2). Both lower (20 μg), and particularly higher, levels of Chl (70 and 100 μg) negatively affected maximum H_2 photoproduction rates.

However, the total yield of H_2 following 168 h of photoproduction was achieved with 70 μg Chl, with total yields increasing with the amount of Chl up until this point (0.14 to 0.36 mol m^{-2}, Table 2). Further increasing Chl to 100 μg did not improve the total H_2 production yield. Thus, increasing the Chl amount in strips from 40 μg (Figs. 3 and 4) to 70 μg would further enhance the total H_2 production yield of heterocysts immobilized in thin films.

4. Conclusion

This is, to the best of our knowledge, the first report and thus a proof of concept for the immobilization of isolated cyanobacterial heterocysts in thin-layer artificial films as long-term, light-induced nitrogenase based production systems. Production activity was measured as acetylene reduction or H_2 production and was improved and prolonged under the following conditions: (i) strictly anaerobic preparation of heterocysts; (ii) immobilization of heterocysts in thin films containing 70 μg Chl; (iii) addition of 200 mM sucrose (iii); and (iv) the addition of substrate (H_2 or N_2) for the recovery of nitrogenase activity. This latter point indicates the potential for a production platform with improved efficiency via switching between fixed nitrogen or hydrogen production modes and is something we will pursue in the future.

Funding

This work was supported by the Academy of Finland (# 287504 to YA and the Finnish Center of Excellence, project #307335), the Novo Nordisk Fonden (project #NNF16OC0021626), the Nordforsk (Nordic Center of Excellence "NordAqua" #82845).

CRediT authorship contribution statement

Alena Volgusheva: Methodology, Investigation, Validation, Visualization, Writing - original draft. **Sergey Kosourov:** Methodology, Formal analysis, Writing - review & editing. **Fiona Lynch:** Formal analysis, Writing - original draft, Writing - review & editing. **Yagut Allahverdiyeva:** Conceptualization, Methodology, Funding

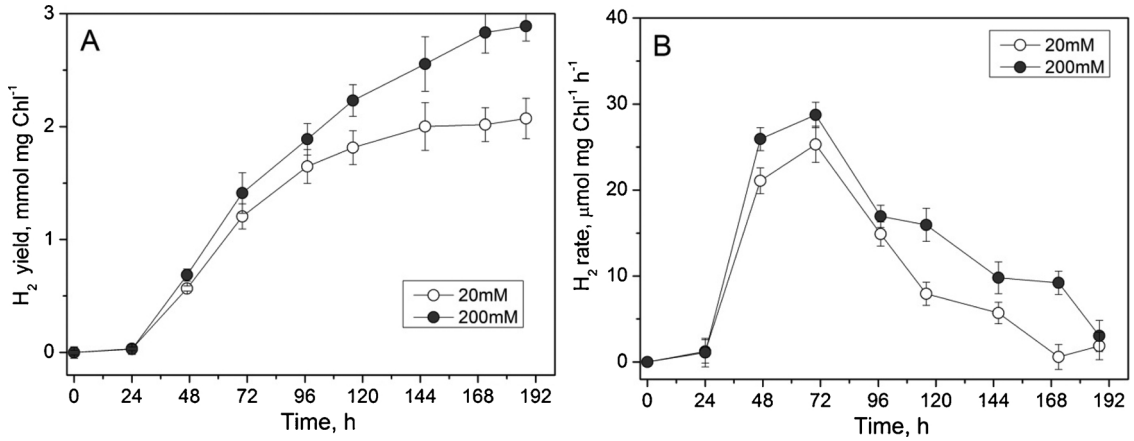

Fig. 3. H_2 photoproduction yield (A) and rate (B) in immobilized heterocysts of *Anabaena* sp. PCC 7120. Heterocysts were placed in medium containing 20 or 200 mM sucrose and incubated in an atmosphere of argon under 70 μmol photons $m^{-2}s^{-1}$ illumination without shaking at 30 °C. The Chl concentration was about 40 μg per strip.

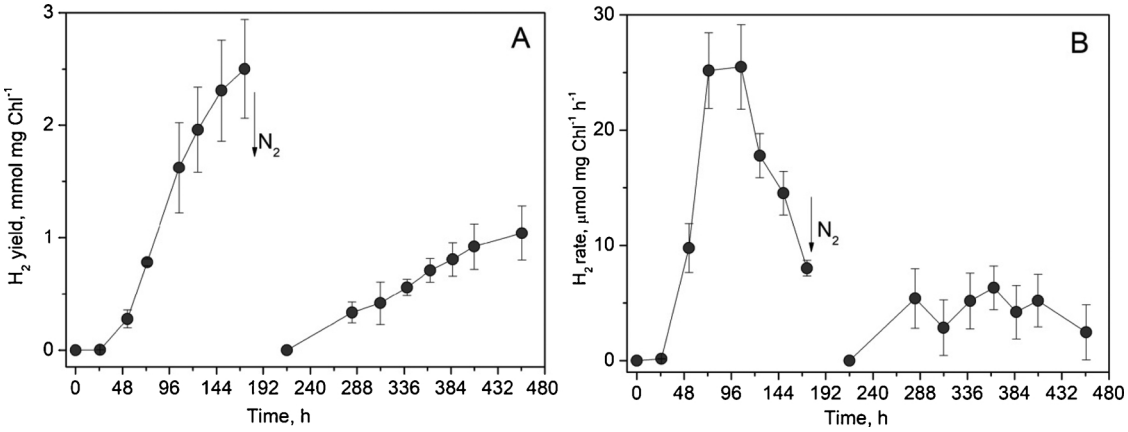

Fig. 4. Effect of N_2 bubbling on yield (A) and rate (B) of H_2 photoproduction in thin-layer immobilized heterocysts. Samples were placed in medium containing 200 mM sucrose and incubated in an atmosphere of argon as described above. Samples were purged with N_2 at 173 h for 20 min, incubated in an atmosphere of nitrogen for 24 h and then purged with argon for 20 min before the next production cycle was started. The Chl concentration was about 40 µg per strip.

Table 2
Effect of Chl on H_2 photoproduction rate and yield in immobilized heterocysts.

Total Chl, µg per strip	The total yield of H_2, mol m^{-2}	The maximum specific rate of H_2 production (µmol mg Chl^{-1} h^{-1})
20 ± 6	0.14 ± 0.04	14 ± 0.9
40 ± 12	0.23 ± 0.03	25 ± 2.5
70 ± 3	0.36 ± 0.04	11 ± 0.6
100 ± 10	0.35 ± 0.01	9 ± 0.7

Samples were incubated in an atmosphere of argon for 168 h. The values represent 3–5 biological replicates.

acquisition, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We thank Dr. M. Ermakova for assistance with heterocyst isolation.

References

Bandyopadhyay, A., Stockel, J., Min, H., Sherman, L.A., Pakrasi, H.B., 2010. High rates of photobiological H2 production by a cyanobacterium under aerobic conditions. Nat. Commun. 1–13.
Bothe, H., Schmitz, O., Yates, M.G., Newton, W.E., 2010. Nitrogen fixation and hydrogen metabolism in cyanobacteria. Microbiol. Mol. Biol. Rev. 74, 529–551.
Brouers, M., Hall, D.O., 1986. Ammonia and hydrogen production by immobilized cyanobacteria. J. Biotechnol. 3, 307–321.
Bui, L.A., Dupre, C., Legrand, J., Grizeau, D., 2014. Isolation, improvement and haracterization of an ammonium excreting mutant strain of the heterocytous cyanobacterium, *Anabaena variabilis* PCC 7937. Biochem. Eng. J. 90, 279–285.
Desimone, M.F., Marzi, M.C., Copello, G.J., Fernández, M.M., Pieckenstain, F.L., Malchiodi, E.L., Díaz, L.E., 2006. Production of recombinant proteins by sol-gel immobilized *Escherichia coli*. Enzyme Microb. Technol. 40, 168–171.
Dickson, D.J., Page, C.J., Elya, R.L., 2009. Photobiological hydrogen production from Synechocystis sp. PCC 6803 encapsulated in silica sol–gel. Int. J. Hydrogen Energy 34, 204–215.
Dilworth, M.J., 1966. Acetylene reduction by nitrogen fixing preparations from *Clostridium pasteurianum*. Biochim. Biophys. Acta 127, 285–294.
Ermakova, M., Allahverdiyeva, Y., 2015. Isolation of Heterocysts from Anabaena sp. PCC 7120. Bio-Protocol 5 (8), e1456. https://doi.org/10.21769/BioProtoc.1456.
Ermakova, M., Battchikova, N., Richaud, P., Leino, H., Kosourov, S., Isojärvi, J., Peltier, G., Flores, E., Cournac, L., Allahverdiyeva, Y., Aro, E.M., 2014. Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Proc. Natl. Acad. Sci. U. S. A. 111, 11205–11210.
Flores, E., Herrero, A., 2010. Compartmentalized function through cell differentiation in filamentous cyanobacteria. Nat. Rev. Microbiol. 8, 39–50.
Gautier, C., Cougnon, C., Pilarda, J.-F., Casse, N., 2006. Label-free detection of DNA hybridization based on EIS investigation of conduction properties of functionalized polythiophene matrix. J. Electroanal. Chem. 587, 276–283.
Grizeau, D., Bui, L.A., Dupré, C., Legrand, J., 2015. Ammonium photo-production by heterocystous cyanobacteria: potentials and constraints. Crit. Rev. Biotechnol. 36, 607–618.
Haselkorn, R., 1978. Heterocysts. Annu. Rev. Plant Physiol. Plant Mol. Biol. 29, 319–344.
Homburg, S.V., Kruse, O., Patel, A.V., 2019. Growth and photosynthetic activity of Chlamydomonas reinhardtii entrapped in lens shaped silica hydrogels. J. Biotechnol. 302, 58–66.
Jensen, B.B., Cox, R.P., Burris, R.H., 1986. Isolation of cyanobacterial heterocysts with high and sustained dinitrogen-fixation capacity supported by endogenous reductants. Arch. Microbiol. 145, 241–247.
Khadka, N., Milton, R.D., Shaw, S., Lukoyanov, D., Dean, D.R., Minteer, S.D., Raugei, S., Hoffman, B.M., Seefeldt, L.C., 2017. Mechanism of nitrogenase H_2 formation by metal-hydride protonation probed by mediated electrocatalysis and H/D isotope effects. J. Am. Chem. Soc. 139, 13518–13524.
Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microb. Cell Fact. 17, 36.
Kosourov, S., Murukesan, G., Seibert, M., Allahverdiyeva, Y., 2017. Evaluation of light energy to H_2 energy conversion efficiency in thin films of cyanobacteria and green alga under photoautotrophic conditions. Algal Res. 28, 253–263.
Kosourov, S.N., Seibert, M., 2009. Hydrogen photoproduction by nutrient-deprived Chlamydomonas reinhardtii cells immobilized within thin alginate films under aerobic and anaerobic conditions. Biotechnol. Bioeng. 102, 50–58.
Kumar, A., Tabita, R., Van Baalen, C., 1982. Isolation and characterization of heterocysts from Anabaena sp. Strain CA. Arch. Microbiol. 133, 103–109.
Laurens, L.M.L., Markham, J., Templeton, D.W., Christensen, E.D., Van Wychen, S., Vadelius, Eric W., Melodie Chen-Glasser, Dong, T., Davis, R., Pienkos, P.T., 2017. Development of algae biorefinery concepts for biofuels and bioproducts; a perspective on process-compatible products and their impact on cost-reduction. Energy Environ. Sci. 10, 1716.
Leino, H., Kosourov, S.N., Saari, L., Sivonen, K., Tsygankov, A.A., Aro, E.-M., Allahverdiyeva, Y., 2012. Extended H2 photoproduction by N2-fixing cyanobacteria immobilized in thin alginate films. Int. J. Hydrogen Energy 37, 151–161.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.
Lips, D., Schuurmans, J.M., dos Santos, F.B., Hellingwerf, K.J., 2018. Many ways towards 'solar fuel': quantitative analysis of the most promising strategies and the main challenges during scale-up. Energy Environ. Sci. 11, 1716.
Lockau, W., Peterson, R.B., Wolk, C.P., Burris, R.H., 1978. Modes of reduction of nitrogenase in heterocysts isolated from Anabaena species. BBA-Bioenergetics 502, 298–308.
Lopez-Igual, R., Picossi, S., López-Garrido, J., Flores, E., Herrero, A., 2012. N and C control of ABC-type bicarbonate transporter Cmp and its LysR-type transcriptional regulator CmpR in a heterocyst-forming cyanobacterium, Anabaena sp. Environ. Microbiol. 14, 1035–1048.
Magnuson, A., 2019. Heterocyst thylakoid bioenergetics MDPI. Life 9, 13.
Martín, A.J., Shinagawa, T., Pérez-Ramírez, J., 2018. Electrocatalytic reduction of nitrogen: from Haber-Bosch to ammonia artificial leaf. Chemistry 75, 6271–6275.
Masukawa, H., Zhang, X., Yamazaki, E., Iwatam, S., Nakamura, K., Mochimaru, M., Noue, K., Sakurai, H., 2009. Survey of the distribution of different types of nitrogenases and hydrogenases in heterocyst-forming cyanobacteria. Mar. Biotechnol. 11, 3.
Muro-Pastor, A.M., Hess, W.R., 2010. Heterocyst differentiation: from single mutants to global approaches. Trends Microbiol. 20, 548–557.
Musgrave, S.C., Kerby, N.W., Codd, G.A., Stewart, W.D., 1982. Sustained ammonia production by immobilized filaments of the nitrogen-fixing cyanobacterium Anabaena

27893. Biotechnol. Lett. 4, 647–652.
Nieto, I., Rocchietti, S., Ubiali, D., Speranza, G., Morelli, C.F., Fuentes, I.E., Alcantara, A.R., Terreni, M., 2005. Immobilization of different protein fractions from Rhizomucor miehei lipase crude extract - enzymatic resolution of (R,S)-2-tetralol. Enzyme Microb. Technol. 37, 514–520.
Peterson, R., Wolk, C.P., 1978. High recovery of nitrogenase activity and of 55Fe-labeled nitrogenase in heterocysts isolated from *Anabaena variabilis*. Proc. Natl. Acad. Sci. U. S. A. 75, 6271–6275.
Pressi, G., Toso, R.D., Monte, R.D., Carturan, G., 2003. Production of enzymes by plant cells immobilized by sol-gel silica. J. Sol-Gel Sci. Technol. 26, 1189.
Razquin, P., Bes, M.T., Peleato, M.L., Fillat, F., 2002. Monitoring heterocyst isolation in *Anabaena* PCC 7119. J. Plant Biochem. Biotechnol. 11, 53–55.
Thiel, T., Pratte, B., 2014. Regulation of three nitrogenase gene clusters in the cyanobacterium *Anabaena variabilis* ATCC 29413. Life (Basel) 4, 944–967.
Walsby, A.E., 2007. Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange. Trends Microbiol. 15, 340–349.
Wolk, C.P., Ernst, A., Elhai, J., 1994. Heterocyst metabolism and development. In: Bryant, D.A. (Ed.), The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 769–823.
Wolk, C.P., 2000. Heterocyst formation in Anabaena. In: Brun, Y.V., Shimkets, L.J. (Eds.), Prokaryotic Development. American Society for Microbiology, Washington, D.C, pp. 83–104.
Wutthithien, P., Lindblad, P., Incharoensakdi, A., 2019. Improvement of photobiological hydrogen production by suspended and immobilized cells of the N_2-fixing cyanobacterium Fischerella muscicola TISTR 8215. J. Appl. Phycol. 1–10.
Zhang, Y.H., Zhang, Y.L., Shen, Q.R., 2007. Nitrogen accumulation and translocation of different Japonica rice cultivars under different nitrogen application rates. Pedosphere 17, 792–800.