Photosynthesis driven continuous hydrogen production by diazotrophic cyanobacteria in high cell density capillary photobiofilm reactors

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Abstract

Hydrogen (H_2) is a promising fuel in the context of climate neutral energy carriers and photosynthesis-driven H_2-production is an interesting option relying mainly on sunlight and water as resources. However, this approach depends on suitable biocatalysts and innovative photobioreactor designs to maximize cell performance and H_2 titers. Cyanobacteria were used as biocatalysts in capillary biofilm photobioreactors (CBRs). We show that biofilm formation/stability depend on light and CO_2 availabilityH_2 production rates correlate with these parameters but differ between *Anabaena* and *Nostoc*. We demonstrate that high light and corresponding O_2 levels influence biofilm stability in CBR. By adjusting these parameters, biofilm formation/stability could be enhanced, and H_2 formation was stable for weeks. Final biocatalyst titers reached up to 100 g l^{-1} for *N. punctiforme* ATCC29133 NHM5 and *Anabaena* sp. PCC7120 AMC 414. H_2 production rates were up to 300 µmol H_2 l^{-1} h^{-1} and 3 µmol H_2 g_{cdw}^{-1} h^{-1} in biofilms.

Keywords:

Photosynthesis driven hydrogen production

Capillary biofilm photobioreactors

Cyanobacteria

Introduction

Molecular hydrogen (H_2) is an important energy carrier for a future, non-fossil energy landscape. Different concepts for sustainable H_2 production have been described (Bühler et al., 2021). The main focus is currently on water electrolysis driven by renewable energy derived, e.g., from wind or sun. However, electrolysis depends on nobel metal catalysts with limited availability. Photosynthesis-driven H_2 production is potentially carbon-neutral, relying on sunlight, abundant salts, and water as major resources for biocatalyst operation (Khetkorn et al., 2017; Tiwari & Pandey, 2012). Since several decades, researchers work on wiring H_2 production to the photosynthetic apparatus of cyanobacteria or green algae performing oxygenic photosynthesis (Krishnan et al., 2018; Martin & Frymier, 2017). Nitrogenases and hydrogenases are the most promising enzymes to make use of photosynthesis-derived reduction equivalents for H_2 formation (Bothe, 2016). However, the pronounced O_2 sensitivity of most of these enzymes hamper biotechnological applications, especially when applied in phototrophs performing oxygenic photosynthesis. Current approaches for H_2 production involve either the (temporary) establishment of anaerobic conditions or the use of diazotrophic cyanobacteria forming heterocysts as anaerobic reaction compartments for operating O_2-sensitive enzymes. Yet, both approaches suffer from an indirect and thus inefficient coupling of H_2 formation to photosynthesis. The advantage of diazotrophic cyanobacteria is their independence of cost-intensive nitrogen source feeding. This may enable low production costs and could also provide valuable biomass with high N content for other applications like animal feed or fertilizers (Pathak et al., 2018).

Conventional photobioreactor systems are often limited in productivity by low biomass concentrations due to illumination efficiency, gas mass transfer (especially

low carbon availability), product removal, nutrient supply, and relatively high operation costs (Posten, 2009). These features typically result in slow cell growth, low cell densities, and short process times with narrow production windows and low productivities (Fernandes et al., 2015; Fu et al., 2019; Hariskos & Posten, 2014; Johnson et al., 2018; Kirnev et al., 2020). New cultivation concepts to achieve high, stable, and potentially scalable productivities for the phototrophic production of biomass, chemicals, and fuels involve, e.g., cell retention / immobilization in membrane photobioreactors or biofilm photobioreactors (Bähr et al., 2016; ; Li et al., 2019; Podola et al., 2017; Schultze et al., 2015). In addition, several studies demonstrated that artificial or natural biofilms enable enhanced and prolonged biomass production and product formation (Schultze et al., 2015; Zhang et al., 2017). However, artificial cell immobilization is limited by the stability of encapsulated cells (Homburg et al., 2019; Vorndran & Lindberg, 2016). Capillary photobiofilm reactors, CBRs, based on natural biofilm formation, were reported as a promising solution, as previously shown for photosynthesis-driven oxyfunctionalization of cyclohexane to cyclohexanol (Hoschek et al., 2019). The capillary biofilm reactor concept set a benchmark regarding achievable biomass concentration (up to 58 g_{cdw} L^{-1}) and productivity for selective C-H hydroxylation (up to 3.76 g cyclohexanol m^{-2} day^{-1}) applying recombinant *Synechocystis* sp. PCC 6803 cells as biocatalysts. Recently, the application was extended to other cyanobacteria, demonstrating the potential and importance of a photo- and heterotrophic co-cultivation and the possibility to use diazotrophic cyanobacteria with nitrogen free media (Bozan et al., 2022) for the production of H_2. Several studies showed that additional factors define H_2 production, namely biofilm thickness and biofilm structure (Liao et al., 2015), which in turn are influenced by surface properties of the capillaries and can be tuned by applying special coatings (Li et al., 2017). Capillary biofilm bioreactors have also been applied

to concepts based on dark fermentation (Renaudie et al., 2021) and combined approaches of dark fermentation and photo- H_2 production (Cheng et al., 2022).

Here, the suitability of CBRs for continuous H_2 production with diazotrophic cyanobacteria was investigated. Up to date, the highest productivities for H_2 formation in light and thus in the presence of photosynthetic water oxidation are reported for diazotrophic filamentous cyanobacteria. These microbes produce H_2 as a side product during N_2 fixation in heterocysts. In the natural system, the energy in the produced H_2 is metabolically recycled via an uptake hydrogenase. In the two model strains employed in this study, the respective uptake hydrogenases have been deleted, although *Anabaena* sp. PCC 7120 still contains a bidirectional hydrogenase. However, it was shown that H_2 production is typically not impaired by this enzyme (Masukawa et al., 2002).

The productivity of the CBR reactor regarding H_2 formation was quantified. Importantly, the impact of parameters like light intensity and O_2 concentration on biofilm development and stability as well as H_2 productivity was investigated. Two filamentous, diazotrophic cyanobacteria, *Nostoc punctiforme* ATCC 29133 NHM5 and *Anabaena* sp. PCC 7120 AMC 414 lacking H_2 uptake hydrogenases showed high H_2 production rates in continuous capillary photobioreactors. This study highlights future strain and reactor engineering targets paving the way for efficient photosynthesis-driven H_2 production using biocatalysts based on biofilm forming cyanobacteria.

Material and Methods

Strains & shake flask cultivation

The two uptake hydrogenase-deficient filamentous diazotrophic cyanobacterial strains, *Nostoc punctiforme* ATCC 29133 NHM5 (Δ*hupL*), hereafter called NHM5, and

Anabaena sp. PCC 7120 AMC 414 (Δ*xisC*, recombinase), hereafter named AMC 414, and the corresponding wild type strains were investigated (see the list of strains used in this study, Table 1). All strains were cultivated in BG-11 medium (buffered 10 mM HEPES; pH 7.2)(with nitrate) (Lindberg et al., 2002) at 30°C under continuous white light (25 µmol photons m^{-2} s^{-1}) in Erlenmeyer flasks in a multitron shaker (Infors, Bottmingen, Switzerland) prior to inoculation in CBRs, as described previously (Figure 1) (Heuschkel et al., 2019b; Hoschek et al., 2019). *Pseudomonas taiwanensis* VLB 120 was cultivated as described previously and mixed in a defined ratio prior to biofilm inoculation and cultivation (Bozan et al., 2022).

Capillary biofilm reactor operation

Polystyrene capillaries (25 cm length, 3 mm inner diameter, 1.76 ml) were used as bioreactor units. The capillary diameter was intentionally selected to obtain a high surface area to volume ratio (1333 m^2 m^{-3}) and low light penetration depth. In addition, the described aqueous-air slug flow enables Taylor flow conditions beneficial for maximizing the mass transfer of heat, nutrients and gaseous compounds. As shown previously, (Hoschek et al, 2019), these conditions stabilize biofilm thickness, prevent congestion and prevent limiting mass transfer of nutrients and gases. Polystyrene was selected as material, to minimize diffusion-related H_2 loss. Defined mixtures of *P. taiwanensis* VLB 120 and the cyanobacterial strains were inoculated as described for initial biofilm formation (Hoschek et al., 2019). Operating the CBRs in a segmented flow regime (Figure 1), BG-11 medium and air were applied at an equal flow rate of 52 µl min^{-1} using a peristatic pump (Ismatec, Wertheim, Germany) under continuous illumination at room temperature (air-conditioned to 25°C). H_2 production was induced by switching to diazotrophic conditions (applying nitrogen-free BG-11_0 medium). Keeping the gas flow constant, the gas composition was varied, reducing the O_2

concentration by replacing air with argon or nitrogen via a gas-tight balloon filled with the respective gas and connected to the airflow line. The gas composition in the CBRs was measured as described below. The biofilm dry weight was determined at the end of the experiments as described previously (Hoschek et al., 2019). Light was applied with a LED light system (Cell Deg, Berlin, Germany) at intensities ranging from 25 to 150 µmol photons m^{-2} s^{-1}. It is important to note that the LED provided blue (460 nm) and red light (660 and 680 nm), being photosynthetically more effective than white light. Sodium bicarbonate (Sigma-Aldrich) was added to the media (0-20 mM) as a carbon source.

H_2 – quantification / analysis

Custom-made bubble traps were connected to the outlet of the cultivation unit for H_2 and O_2 quantification. The bubble trap system (1 ml total volume) was flushed with air after every sampling of the gas phase (5 ml). A gas-tight syringe (Hamilton, Reno, NV) was inserted into the bubble trap, and 100 µL gas phase were withdrawn and manually injected into a gas chromatograph (Thermo scientific, Trace1310 equipped with a TG Bond MIsieve 5A column with 0.32 mm inner diameter and 0.20 µm film thickness). The thermal conductivity detector and the oven were adjusted to 100 and 75°C, respectively. Gas concentrations were calculated based on calibration curves determined with defined gas mixtures. Volumetric and biomass specific H_2 production rates were calculated based on the percentage of H_2 in the gas phase, the flow rate, and the biomass in the capillaries.

H_2 production by all strains was additionally determined for cell suspensions in closed vials. Cells were cultivated in BG-11 medium as described above. H_2 production was induced by transferring the cells into $BG\text{-}11_0$ medium. To this end, cells harvested by centrifugation (4000g, room temperature) were washed twice with $BG\text{-}11_0$ medium

followed by resuspension in the same medium. Upon continued incubation in Erlenmeyer flasks, cell suspension samples (5 mL) were taken 24, 48, and 72 h after the transfer to BG-11_0 medium, transferred into sealed GC vials (Thermo Scientific), supplied with 10 mM sodium bicarbonate, and incubated for 24 h at 30°C under continuous light (50 µmol photons m^{-2} s^{-1}) to quantify H_2 production. H_2 formation rates were determined by GC analysis of the gas phase as described above.

Results and discussion

H_2 production in planktonic cultures

H_2 production of all strains (*Nostoc punctiforme* ATCC 29133 WT, *Nostoc punctiforme* ATCC 29133 NHM5, *Anabaena* sp. PCC 7120 WT, *Anabaena* sp. PCC 7120 AMC 414) was first tested in planktonic cultures. Cell suspensions in BG-11_0 (5 ml) were incubated in a multitron cultivation chamber (Infors) in closed 20 ml GC vials (Thermo Scientific) under illumination (25 µmol photons m^{-2} s^{-1}). H_2 formation rates were determined 24, 48, and 72 h after transition into nitrogen-free BG-11_0 medium. Samples taken at these time points were incubated in sealed vials, and H_2 accumulation was analyzed (see materials and methods section for details). For NHM5, high activities were measured after 24 h indicating heterocyst development, whereas this was found to take 48 h in the case of AMC 414. The hydrogenase-deficient strains showed 5 times higher biomass specific H_2 formation rates compared to the corresponding wild type (WT) strains. NHM5 showed the highest specific H_2 formation rate (15 µmol H_2 g_{cdw}^{-1} h^{-1}, with an average rate of 12±3,5 µmol H_2 g_{cdw}^{-1} h^{-1}rates measured for samples taken after 24, 48, and 72 h). For AMC 414, this highest rate was 12 µmol H_2 g_{cdw}^{-1} h^{-1}, 48 h after nitrogen deprivation (average: 8±4 µmol H_2 g_{cdw}^{-1} h^{-1}). Assuming that Chl a accounts for 1% of the cell dry weight (Zavrel et al., 2019), these rates translate into 1.5 and 1.2 µmol H_2 (mg Chl a)$^{-1}$ h^{-1}, respectively.

H_2 production in capillary biofilm photobioreactors

Biofilm development of *N. punctiforme* ATCC 29133, *Anabaena* sp. PCC 7120, and their respective hydrogenase-deficient mutants was analyzed in capillary biofilm photobioreactors (CBRs) as a first step towards biofilm-based H_2 production. Co-cultivation with *P. taiwanensis* VLB120 has been reported to promote biofilm formation with the cyanobacterial strains *Synechocystis* sp. PCC 6803 (Heuschkel et al., 2019b; Hoschek et al., 2019) and *Tolypotrix* sp. PCC 7712 (Bozan et al., 2022). Similarly, only very poor or no biofilm formation was detected for monoseptic CBR cultures of NHM5, AMC 414, and respective wildtype strains, whereas good biofilm growth was obtained upon co-cultivation with *P. taiwanensis* VLB120 (data not shown). Thereby, no addition of an organic carbon source was necessary. Uptake hydrogenase deletion strains and respective WT strains showed identical growth behaviors. Biofilm formation was slightly enhanced in terms of surface coverage upon nitrogen deprivation resulting in biomass concentrations of 8-10 g_{cdw} l^{-1}. The optimal initial cell ratio of cyanobacteria and heterotrophic partner was determined to be 1:1, enabling optimal biofilm formation and minimal biofilm detachment. It is important to note that the heterotrophic cells are mainly needed for initial biofilm formation. The final fraction of *Pseudomonas* cells in the biofilm is very low, especially, when no organic carbon and energy source is added (Heuschkel et al. 2019a, Bozan et al. 2022), like in the process reported here. Therefore, cyanobacterial cells are the main species in the biofilm in the final stadium. Neither *N. punctiforme* WT nor *Anabaena* WT produced H_2 during CBR cultivations, whereas the respective H_2 uptake-deficient strains in co-culture with *P. taiwanensis* VLB120 showed H_2 production. It is important to note that the determined biomass consists of both species in all biofilm experiments, whereas the heterotrophic cells were present in low amounts, see also Bozan et al. (2022). Average H_2 production rates amounted to 72 ± 22 (NHM5) and 150 ± 47 µmol H_2 l^{-1} h^{-1} (AMC 414) for 14 days

(Figure 2). However, the daily H_2 formation rates varied significantly, and biofilms remained patchy in terms of surface coverage (see supplemental material). Remarkably, these rates correspond to similar specific rates as measured for suspended cells, for which, however, H_2 formation rates may not have been constant in the analyzed 24 h time range.

The poor surface coverage in the first CBR experiments indicated that growth and H_2 production were limited. So, what parameters are basically determining growth, biofilm formation and the H_2 formation rate? After initial biofilm formation, sodium bicarbonate supply (10 mM) to the BG-11_0 medium was tested and indeed improved biofilm formation and H_2 production (Figure 3 (I)). Both uptake hydrogenase-deficient strains showed enhanced growth and surface coverage, which, however, was still lower than described for *Synechocystis* sp. PCC 6803 (Hoschek et al., 2019). H_2 formation rates with both strains increased in the beginning and were then stable for several days with 300 µmol H_2 l^{-1} h^{-1} for NHM5 and 75 µmol H_2 l^{-1} h^{-1} for AMC 414. NHM5 produced 4 times more H_2 compared to AMC 414 and to conditions without carbonate, whereas AMC 414 produced only half the amount of H_2 compared to conditions without carbonate. To test whether H_2 production is still limited by carbon availability, the sodium bicarbonate concentration was increased to 20 mM (Figure 3 (II)). However, biofilm stability with NHM5 was impaired and detachment events occurred. H_2 production with NHM5 dropped to 150 µmol H_2 l^{-1} h^{-1}. For AMC 414, a slight increase in H_2 production was observed (up to 100 µmol H_2 l^{-1} h^{-1}). Reestablishing a $NaHCO_3$ concentration of 10 mM in the medium inflow and increasing the light intensity from 25 to 75 µmol photons m^{-2} s^{-1} led to enhanced biofilm formation, i.e., the establishment of dark green biofilms covering the whole capillary (Figure 3 (III)). Especially AMC 414 showed an increased H_2 production with up to 150-200 µmol H_2 l^{-1} h^{-1}, to a comparable

level as obtained with NHM5. After 70 days a reduced H_2 production was measured for both strains and to avoid flush-outs the experiment was stopped. Most likely, the elevated light and CO_2 availability caused an increase in O_2, and a reduction in cell fitness, leading to a biofilm instability. This is supported by experiments with reduced O_2 concentrations (Figure 4), showing that reduction of O_2 can increase biofilm stability and H_2 production. In total, both investigated uptake hydrogenase-deficient strains produced H_2 under all conditions for 70 days in continuous culture mode with biofilm-mediated biomass retention (Figure 3). In these reaction setups, the maximal H_2 formation rate amounted to 300 µmol H_2 l^{-1} h^{-1} and an average rate of 250 µmol H_2 l^{-1} h^{-1} was obtained for both strains under respective optimal conditions. The final biomass density with up to 100 g_{cdw} l^{-1} is one of the highest biomass concentrations achieved in a photobioreactor so far and the specific H_2 production rates calculated for the last time interval averaged between 2-4 µmol H_2 g_{cdw}^{-1} h^{-1}. The biomass concentration in the capillaries is highly dynamic, depending on the cultivation conditions. Thus, specific rates in the earlier stages in the experiments shown in Figure 3, when cell concentrations were lower (in the range between 10 to 100 g_{CDW} L^{-1}), can be assumed to have been higher. As a consequence, the given specific activity range of 2-4 µmol H_2 g_{cdw}^{-1} h^{-1} constitutes a lower boundary.

The possibility to utilize diazotrophic cyanobacteria in CBRs for H_2 production and possible hurdles to be overcome for sustainable photosynthesis-driven H_2 production was investigated. Previously, it has been shown that CBRs can be utilized to cultivate a range of cyanobacteria, including dioazotrophic cyanobacteria. Especially *Tolypotrix* sp. PCC 7712 showed high potential to be applied in CBRs (Bozan et al., 2022). However, genetic accessibility, e.g., to knock out uptake hydrogenases, favors other strains like *Nostoc punctiforme* ATCC 29133 NHM5 (Δ*hupL*) and *Anabaena* sp. PCC

7120 AMC 414 (Δ*xisC*, recombinase). In good accordance with Bozan et al. (2022), the experiments show that biofilm formation with these strains profits from the presence of a heterotrophic biofilm building strain like *P. taiwanensis*. Further, biofilm formation was significantly better in nitrogen free- as compared to normal BG-11 medium.

Furthermore, it was shown that light and elevated carbon availability improved biofilm formation and, associated with it, H_2 production. Several studies demonstrated that both strains produce H_2 in flask and bioreactor experiments under batch cultivation conditions (Lindberg et al., 2002, Lindblad et al., 2002, Masukawa et al., 2002, Avilan et a., 2018). The time range, in which H_2 is produced could be significantly extended to 70 days with improved cultivation conditions. It was possible to produce H_2 continuously over weeks. Previous studies showed short production phases of maximally 30 days (Kosourov et al., 2014; Touloupakis et al., 2016) under batch conditions. Ethylene, also a gaseous product, was recently produced in a similar biofilm-based approach for 38 days (Vajravel et al., 2020). In accordance with the presented results, the authors could also demonstrate that product formation depends on light and carbon availability.

The biomass concentrations reached in the CBRs are among the highest reported for cyanobacteria in bioreactors. Compared to conventional photobioreactors with biomass densities up to 20 $g_{cdw}\ l^{-1}$ (Bähr et al., 2016) and other biofilm photobioreactor designs (up to 60 $g_{cdw}\ l^{-1}$, Wang et al., 2017), the reported CBR setup enabled the highest biomass concentration (100 $g_{cdw}\ l^{-1}$) reported so far for phototrophic microorganisms. One the other hand, with a different purpose, i.e., production of biomass, value-added products therein, or waste water treatment, only porous substrate-based photobioreactors (PSBRs) showed a higher biomass production up to 300 $g_{cdw}\ l^{-1}$ (Podola et al., 2017). Yet, in PSBRs, biofilms are not in direct contact with

cultivation liquid, and normalizing biomass amounts produced to the cultivation liquid volume is complex, with productivities and economics for biomass harvest rather to be calculated relative to the area as g dry mass per m^2 and day (Li et al., 2019). In addition, efficient applications of PSBRs for extracellular chemical or biofuel products are not reported and seem to be challenging, due to product evaporation, mass transfer, and biofilm ageing. This is especially the case for gaseous products.

Biofilm formation and H_2 production is light dependent

The experiment shown in Figure 3 indicated a significant influence of the light intensity on H_2 formation and surface coverage of biofilms of cyanobacteria in consortia with Pseudomonads. Thus, the light dependency of H_2 formation by NHM5 and AMC 414 was analyzed further. The CBRs were operated as described above with air segments in a slug flow mode. Cultivation and H_2 formation were tested at five different light intensities (50, 75, 100, 125, and 150 µmol photons m^{-2} s^{-1}) with 10 mM $NaHCO_3$ in the medium. Each light intensity was applied for five days and the corresponding H_2 formation rates were determined (Figure 4 A+B). For NHM5-based biofilms, volumetric H_2 formation rates increased with the light intensity up to 125 photons m^{-2} s^{-1} to a maximum of 75 µmol H_2 l^{-1} h^{-1}. It is important to note that volumetric production rates were lower compared to the long-term experiments, since final biomass concentrations were lower (between 30-50 g_{CDW} l^{-1}). A further increase in light intensity led to reduced H_2 formation accompanied by increasing O_2 concentrations. At high light intensities, cells and biofilm fragments detached from the capillaries, were flushed out, and consequently H_2 production stopped (data not shown). It is important to note that biofilms recovered in a few days inside the capillaries after the light intensity was reduced again to 50 µmol photons m^{-2} s^{-1}. Also, AMC 414 biofilms showed increased H_2 production with increasing light intensity. Production rates increased from 35 to 75

318 µmol H_2 l^{-1} h^{-1} at 150 µmol photons m^{-2} s^{-1}. At high light intensity, biofilm detachment
319 was observed as for NHM5. Previously, David et al., 2015 estimated light attenuation
320 within a cyanobacterial biofilm. Theoretically, for a light intensity of 150 µmol photons
321 m^{-2} s^{-1} and a light attenuation coefficient of 9 mm^{-1}, a maximal biofilm thickness of 250
322 µm can be achieved. It is not clear, how far light can penetrate into biofilm. In natural
323 microbial mats, cyanobacterial layers can reach several mm. This indicates, that there
324 is enough light available also in deeper layers. Furthermore, there are reports on
325 *Synechocystis*, which hypothesize cells acting as a kind of micro-lens, transporting
326 light from one end of the cell to the other and thereby guide it to deeper levels of
327 biofilms (Schuergers et al 2016). In our experiments, light was supplied only from the
328 top, but the capillary surface was covered completely (also the bottom part) indicating,
329 that in this range enough light is available to allow complete surface coverage of the
330 capillary.

331 Besides H_2, O_2 was monitored, as elevated O_2 concentrations are known to impair cell
332 vitality and nitrogenase activity (Zhao et al., 2007). Air segments contained 20.9% O_2
333 at the inflow. During cultivation at low light intensities, O_2 concentrations were constant
334 between 18-20% after 10 min of residence time, whereas high light intensities led to
335 O_2 levels up to 23.5% in the gas phase. It is important to note that gas diffusion though
336 the tubes cannot be excluded. The high light in combination with elevated O_2
337 concentrations and the risk of ROS formation might contribute to the destabilization
338 and final detachment of the biofilm at higher light intensities.

339 To further elucidate the correlation of elevated O_2 concentrations and biofilm
340 detachment, N_2 and Argon instead of air have been applied to the CBRs in segmented
341 flow mode at a constant illumination of 100 µmol photons m^{-2} s^{-1} (Figure 4 C+D). The
342 overall O_2 concentration in the CBR was thus lowered and only 15 – 20 % of O_2 was

343 measured in the gas phase at the outlet of the different set-ups. It is important to note
344 that no completely anaerobic and N_2-depreviated conditions were established as the
345 medium feed was air-saturated and gas diffusion though the tubes cannot be excluded.
346 The changed gas stream stabilized the biofilms in the CBRs and no biofilm
347 detachments were observed, indicating that increased O_2 concentrations at elevated
348 light intensities promoted biofilm detachment. In the case of AMC 414, H_2 production
349 rates slightly decreased upon replacement of air with N_2 or argon. However, NHM5
350 showed a 50% increase in H_2 production when supplied with argon. It is important to
351 note that argon was expected to increase H_2 formation, as this has been found before
352 in short term experiments (Wilson et al., 2021), but would result in a limited N_2 fixation.
353 Here, a sufficient N_2 supply was assured via the medium feed. GC measurements
354 showed a decrease to 40% at the outflow, instead of 80% N_2 in air.

355 The results demonstrated that light and CO_2 availability are main factors determining
356 H_2 production rates in diazotrophic cyanobacteria, since H_2 production is directly linked
357 to growth in these cells. Also, other nutrients influence H_2 production, but their direct
358 effects on H_2 production are still unclear (Howe et al., 2020; Lindberg et al., 2004).

359 Another important factor in CBRs is the gas supply. Under slug flow conditions based
360 on air segments, the O_2 concentrations in CBRs are typically close to that in air or
361 slightly above. However, high photosynthetic rates induced by high light and elevated
362 CO_2 availabilities are accompanied by elevated O_2 production. As a result of the light
363 stress accompanied by high O_2 levels and most likely ROS (reactive oxygen species)
364 formation biofilm stability is affected. Several studies showed similar results
365 (Heuschkel et al., 2020; Hoschek et al., 2019). Elevated O_2 concentrations can
366 promote ROS production, which may result in biofilm detachment (Heuschkel et al.,
367 2020). For effective bioprocessing with phototrophs in biofilms, measures are needed

368 to remove O_2 efficiently, either by utilizing oxygen free gas segments or by means of
369 oxygen consuming heterotrophic cells (Heuschkel et al., 2020). Since the focus of the
370 study was set to determine photosynthesis-driven H_2 production, any organic carbon
371 source for heterotrophs was omitted. Exchange of the air segments with N_2 or Argon
372 reduced O_2 concentrations in the biofilms/capillaries (below 20%) and stabilized H_2
373 production or even lead to a 50% increase in hydrogen production as shown for NHM5.
374 The capillary photobioreactor in combination with segmented flow, allows long-term H_2
375 production and opens the door towards product removal since gaseous H_2 can be
376 extracted from the reaction environment via the air segments, considering appropriate
377 measures to avoid diffusional losses of this highly diffusive gas.

378 In this study, it was shown that light and CO_2 are limiting factors regarding H_2
379 production (Figure 4), as increased light intensity increases H_2 production. However,
380 the upper limits for PBRs in terms of light intensity have to be determined in the future.

381 Recent studies determined additional factors influencing H_2 production, e.g. biofilm
382 thickness, and methods were reported, e.g. fiber optics, to measure and manipulate
383 thickness (Zhong et al., 2014). In our study the biofilm thickness was controlled by
384 the segmented flow, allowing the development of a stable and dense biofilms, see
385 also (Bozan et al., 2022). Finally, new sensors and methods to manipulate light
386 penetration, to measure H_2 production online in biofilms (Chen et al., 2019; Guo et
387 al., 2011; Li et al., 2017) are designed and could help to determine heterogeneities in
388 biofilms regarding H_2 production and this knowledge can help to improve biofilm
389 development and finally H_2 production.

390 Comparison with H_2 formation rates reported in literature showed that rates
391 determined in this study under continuous biofilm cultivation conditions are lower (~3
392 µmol H_2 g_{cdw}^{-1} h^{-1}, but varying with applied biofilm density) than in suspended batch

cultures (~15 µmol H_2 g_{cdw}^{-1} h^{-1}) and compared to literature (50-100 µmol H_2 g_{cdw}^{-1} h^{-1}). Yet, such batch cultures show a high nitrogenase activity only during the exponential growth phase (Lichtl et al., 1997). However, the biofilm co-cultures showed activity for H_2 formation for several weeks. It has to be pointed out, that one has to differentiate between low and high biofilm densities, the first show a higher activity and the second lower rates, pointing towards more maintenance activity in high-density biofilms and low growth. Likewise, novel and advanced methods will help to determine growth rates in combination with H_2 production rates in a temporally and spatially resolved way (Chen et al., 2019; Guo et al., 2011; Li et al., 2017). Additionally, it is challenging to compare H_2 formation rates directly since rates calculated from short-term assays do not always reflect actual product formation rates achievable in the longer term. Importantly, one has to consider H_2 loss by diffusion through the capillaries, as indicated by the experiments with argon and nitrogen.

The continuous H_2 production for weeks in the capillary photobioreactors with both cyanobacterial strains indicates that nitrogenases are active in diazotrophs and cyanobacteria cells retain their activity over a long time. However, the activity in high cell density biofilms was lower, pointing towards low growth and probably maintenance activity. Diazotrophic cyanobacteria can be utilized to provide a stable anaerobic environment inside a heterocyst cell. Such strains are ideal models for developing technical processes for photosynthesis-driven H_2 production. Furthermore, carbon and nitrogen fixation can be combined with the process of H_2 production thus avoiding additional nitrogen sources in cultivation media. Finally, a (dynamic) heterogeneity of the ratios of different microbial species in catalytic biofilms might be of importance for the productivity of H_2 production and will be subject to future studies.

Suitability of CBRs in the industrial use for H_2 production

Capillary units are used as standard tools in continuous industrial processes for the separation or production of matter up to scales of many thousands of tons or m^3 per year. One principle of increasing product volumes (scaling) of these microreactors is the numbering up of individual reactor units (Dong et al., 2021). Assuming a theoretical numbering up of the reaction units used in the CBR setup based on a monolayer of capillaries next to each other on a total flat area of 100 m * 100 m (1 ha) without considering the actual design or geometry of the final unit: Scale-up of the biofilm photobioreactor (CBR) to 100 m^2 would in theory yield about 10 kg dry biomass, considering a flat single-layer setup with about 13200 reaction units of the CBR setup (25 cm length, 3 mm outer diameter, 1 ml volume). In the present study, mature biofilms with 100 $g_{cdw}\ l^{-1}$ produced on average 3 µmol $H_2\ g_{cdw}^{-1}\ h^{-1}$. Considering 1000 h sunshine a^{-1} as a rough average estimation for Germany, this would correspond to 6 kg H_2 to be produced in an area of 1 hectare in one year. Assuming the same basic reaction setup with a different biocatalyst for H_2 production based on a hydrogenase with a turnover number of 100 s^{-1} instead of the nitrogenase (1 s^{-1}) for H_2 formation might already reach an impressive range for small-scale technical applications. Furthermore, three-dimensional constructions instead of monolayer designs, e.g., artificial, leaf-like, or vertical structures, can be expected to significantly enhance the productivity per area.

Together with the specific biocatalyst activity, several other factors are critical for efficient biotechnological applications, i.e., light penetration, achieved cell densities, energy costs for mixing and cooling, and efficient product recovery. The approach presented here, showed that capillary photobioreactors enable continuous production formats and very high cell densities. Reduction of the O_2 concentration was essential

to ensure biofilm stability. Utilizing artificial gas mixtures reduced O_2 under what can be considered as a critical level. Thus, controlling gas concentrations is crucial for technical applications. Argon atmosphere increases H_2 production by a factor of 4 compared to N_2 fixing conditions (Lindblad et al., 2002). This, however, is not feasible in the long term, since nitrogen fixation is essential for cell survival. In conclusion, beneficial effects regarding biofilm formation and H_2 production could be obtained by manipulating the gas composition of the air segments. In combination with an optimal light and carbon provision, an improved and stable H_2 production in CBRs was achieved in continuous mode over weeks with high biomass concentrations. Other critical factors to be addressed in the future include medium demands, temperature effects, and gas diffusion through capillary materials. Overall, designing catalysts with high activity and realizing scalability are now the major challenges to be addressed.

Conclusion

The results demonstrated the principal feasibility of CBRs to produce gaseous products like H_2. This opens the door for future application, especially H_2 production based on hydrogenases in phototrophic organisms. CBRs can now be utilized to investigate essential parameters regulating biofilm development, structure, stability, and scale-up efficiencies. However, several factors still limit technical application, especially medium recycling and methods for gas separation. As the final production process will occur under natural conditions, also the effects of day/night cycles, temperature variations, etc., have to be investigated. Nevertheless, CBRs allow the photosynthesis driven production of a gaseous product in a continuous mode, with a highly active biomass.

E-supplementary data for this work can be found in e-version of this paper online.

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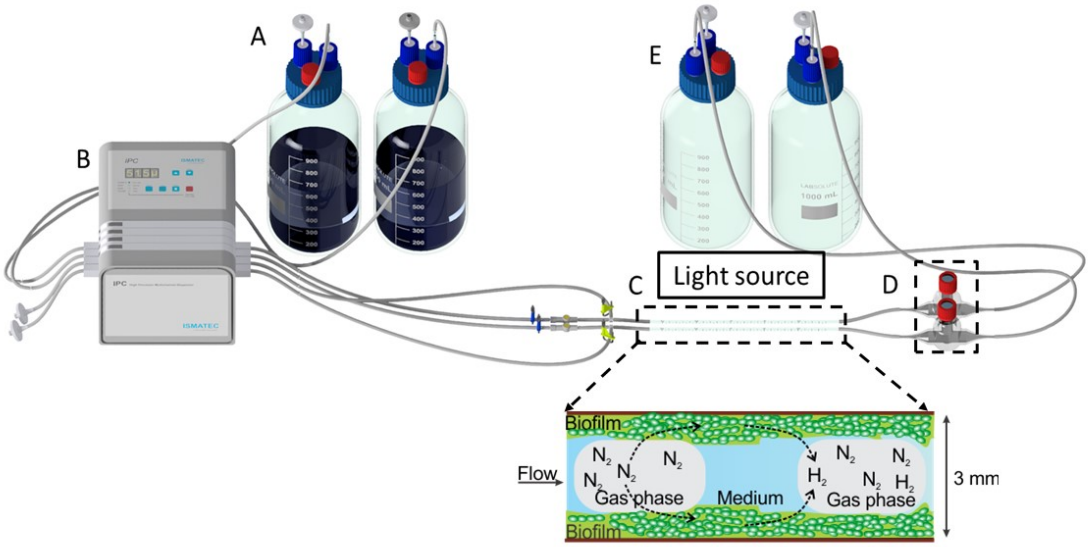
Table 1: Microbial strains used in this study

Strain	Characteristics	
Nostoc punctiforme ATCC 29133	Nitrogen fixing filamentous cyanobacterium	(Lindberg et al., 2002)
Nostoc punctiforme ATCC 29133 NHM5	Uptake hydrogenase knockout of ATCC 29133 (Δ*hupL)*	(Lindberg et al., 2002)
Anabaena sp. PCC 7120	Nitrogen fixing filamentous cyanobacterium	(Lindblad et al., 2002b)
Anabaena sp. PCC 7120 AMC 414	Uptake hydrogenase knockout of PCC 7120 (Δ*xisC*, recombinase)	(Lindblad et al., 2002b)
Pseudomonas taiwanensis VLB120	Biofilm forming strain	(Karande et al., 2016)



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713 **Figure 1.** Setup of the capillary biofilm reactor system. Figure modified from

714 Heuschkel et al. (2019b), see also for details. (**A**) Medium bottles, (**B**) multichannel

715 peristaltic pump, (**C**) cultivation unit, (**D**) bubble trap, (**E**) waste reservoir. The insert

716 depicts a biofilm capillary operated in segmented flow.

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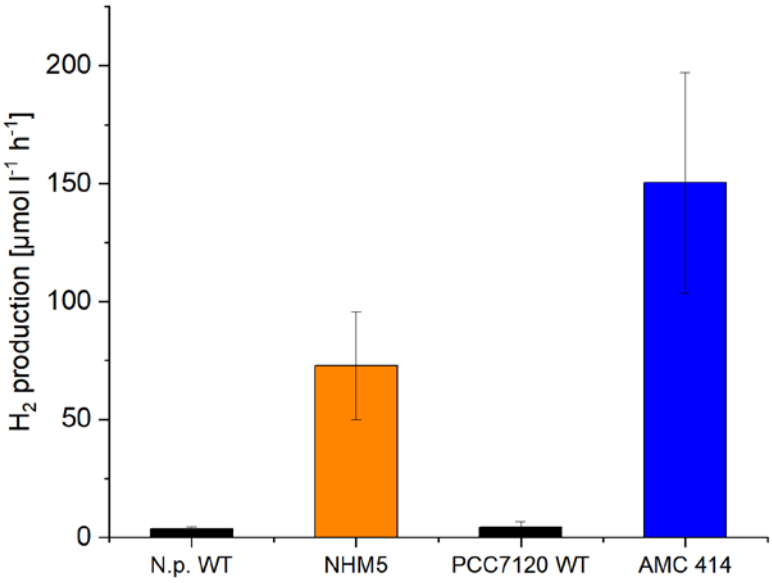
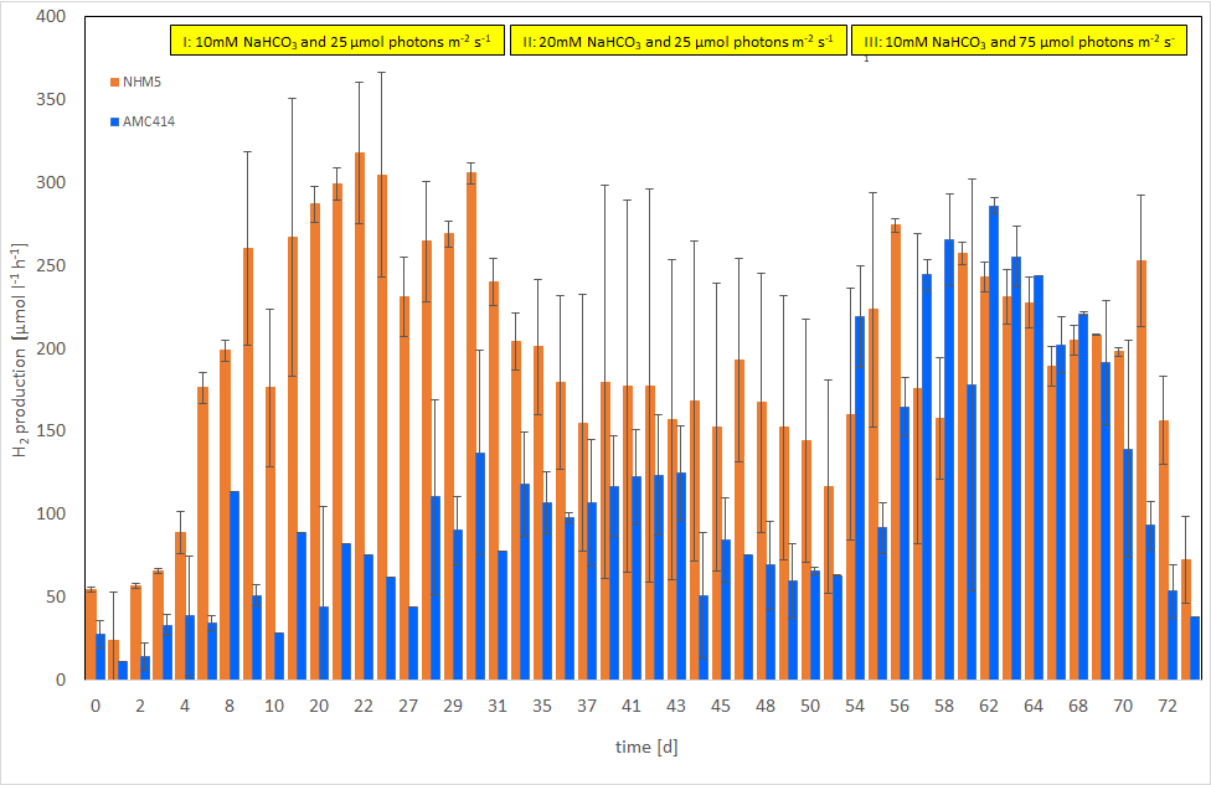


Figure 2: Specific H_2 formation rates of uptake hydrogenase-deficient *N. punctiforme* ATCC 29133 NHM5, *Anabaena* sp. PCC 7120 AMC 414, and respective wildtype strains in capillary photobioreactors. Plotted are the average H_2 production rates in µmol H_2 $l^{-1}h^{-1}$ as measured daily for 14 days in biological duplicates. Biofilms were cultivated as mixed species cultures (with *P. taiwanensis* VLB 120) in 1 ml capillaries under continuous illumination (25 µmol photons m^{-2} s^{-1}) under constant BG-11_0 medium and air flow rates of 52 µl min^{-1}, each.



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736 **Figure 3:** Specific H_2 formation rates of *N. punctiforme* ATCC 29133 NHM5 (orange)
737 and *Anabaena* PCC 7120 AMC 414 (blue) in capillary photobioreactors. Biofilms were
738 cultivated in 1 ml capillaries with 10 mM $NaHCO_3$ in BG-11_0 medium and 25 µmol
739 photons m^{-2} s^{-1} (time window I), 20 mM $NaHCO_3$ and 25 µmol photons m^{-2} s^{-1} (II), and
740 10 mM $NaHCO_3$ and 75 µmol photons m^{-2} s^{-1} (III) for 75 days under a constant medium
741 / air flow of 52 µl min^{-1}. Experiments were performed in duplicates, and H_2
742 concentrations were measured daily as described in Materials & Methods.

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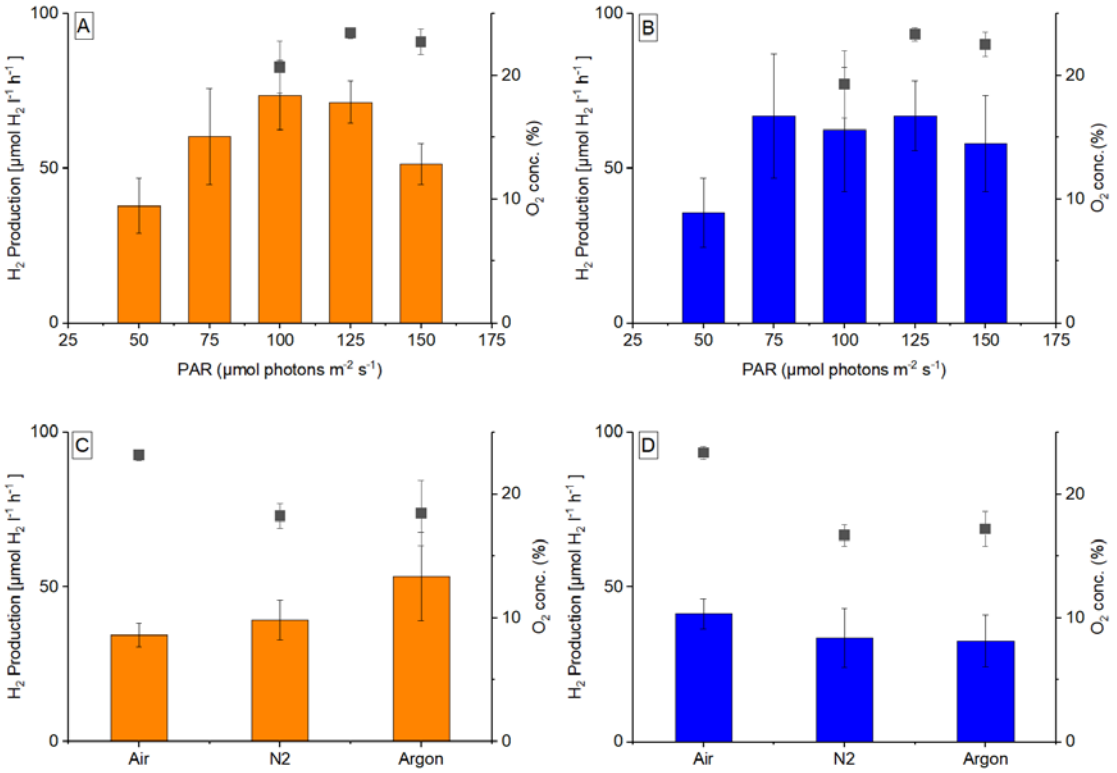

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745 **Figure 4:** H_2 formation of *N. punctiforme* ATCC 29133 NHM5 (A, C, orange) and
746 *Anabaena* PCC 7120 AMC 414 (B, D, blue) in biofilm photobioreactors given as
747 volumetric rates in dependency of the incident light (A, B) and the applied gas phase
748 (C, D). **A+B:** Biofilms were cultivated in 1 ml capillaries at light intensities of 50-150
749 µmol photons m^{-2} s^{-1} in BG-11_0 with 10 mM $NaHCO_3$ under a constant medium and air
750 flow rate of 52 µl min^{-1}, each. Light conditions were kept constant for 7 days, and
751 experiments were performed as biological duplicates. H_2 and O_2 formation were
752 determined daily, and production rates are given as mean values determined on 5
753 consecutive days after an adaptation phase of 2 days. **C+D:** Biofilms were cultivated
754 in 1 ml capillaries under constant illumination of 100 µmol photons m^{-2} s^{-1} in BG-11_0
755 medium with 10 mM $NaHCO_3$. Different gases were provided via the gas segments
756 (Air, N_2, and Argon), and conditions were kept constant for 5 days. H_2 and O_2 formation
757 were measured daily. Experiments were performed in biological duplicates and
758 production rates are shown as mean values.