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Evaluation of self-sustaining cyanobacterial biofilms for technical applications

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ABSTRACT

Cyanobacteria are potent microorganisms for sustainable photo-biotechnological production processes, as they are depending mainly on water, light, and carbon dioxide. Persisting challenges preventing their application include low biomass, as well as insufficient process stability and productivity. Here, we evaluate different cyanobacteria to be applied in a novel capillary biofilm reactor. Cultivated as biofilms, the organisms self-immobilize to the reactor walls, reach high biomass and enable long and robust production processes. As 'best performer' *Tolypothrix* sp. PCC 7712 emerged from this study. It reached the highest biomass in the reactors with $62.6 \pm 6.34\ g_{BDW}L^{-1}$, produced 0.14 μmole H_2 $mg_{Chl\ a}{}^{-1}h^{-1}$ under N_2-fixing conditions, showed optimal surface coverage of the available growth surface, and only minor detachment in contrast to other tested species, highlighting its potential for photobiotechnology in the near future.

1. Introduction

Cyanobacteria are prokaryotes performing oxygenic photosynthesis. In addition, there are many species known for being able of fixing molecular nitrogen. Due to these features cyanobacteria are a highly interesting class of organisms for whole-cell based biocatalysis since they can grow on atmospheric CO_2 (and N_2) as the only source of carbon (and nitrogen), while using water and light for electron and energy supply, respectively. Applied as solar cell factories, they could significantly contribute to future bioeconomy, as they represent potentially sustainable biocatalysts that are independent of organic carbon (and reduced nitrogen compounds) and are able to grow on non-arable land (primacy of food security).

In the last decade, several successful proof-of-principle studies have been published, showing that cyanobacteria can be engineered to produce a wide range of diverse products [1–3]. Nevertheless, application of cyanobacteria on a productive scale remains an exception. Examples are restricted to biomass based processes, like the production of various pigments or applications in the area of biofuel production [4]. Only a handful of species of this very diverse group have been investigated in more detail, including *Synechocystis* sp., *Synechococcus* sp. and *Nostoc* sp.. Persisting challenges like low biomass, low reaction rates, low product titers, and intermittent non-continuous synthesis, prevent their successful industrial scale application [5]. One interesting, novel approach utilizing cyanobacterial biofilms to reach high cell densities has been recently published [6]. Biofilms are surface associated microbial communities embedded in self-produced extracellular polymeric substances (EPS), in which the organisms live in a coordinate fashion whereby they benefit from ecological (micro)niches. The properties of these microbial societies are partially governed by community structure, diffusion of nutrients and extracellular metabolites throughout the biofilm, and the physiological activity of the community [7]. Biofilms are resilient to a wide variety of environmental stresses and are in general less affected by toxic substrates and/or products [8]. Since they are composed of living cells permanently regenerating themselves, biofilms can be regarded as biocatalysts with, in principal, an infinite turn-over number and, therefore, with the potential of establishing continuous bioprocesses. Furthermore, biofilms feature remarkably high cell densities; up to 60 $g_{CDW}L^{-1}$ can be achieved, compared to 4–8 $g_{CDW}L^{-1}$ for suspended cell cultures employing cyanobacteria [5,6]. Despite the fact that cyanobacteria are the primary producers in microbial mats in nature [9], and thus ubiquitous biofilm growing organisms, only little is known about their biofilm forming abilities, especially in technical systems. Studies mostly focus on their role in food webs in an ecological context, investigating natural systems. Recent studies reported that biofilms found in distinct niches often comprehend diverse cyanobacterial species, belonging to the genera *Leptolyngbya*, *Nostoc*, *Synechocystis* and *Tolypothrix* [10,11]. Among these genera, it has been

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Table 1
Cyanobacterial strains used in this study.

Species	Strain used for this study	General remarks about species	References
Cyanothece sp.	PCC 7822	Diurnal diazotrophic unicellular cyanobacterium, genetically accessible, H_2 production	[16,38]
Nostoc punctiforme	ATCC29133	Heterocyst-forming model filamentous cyanobacterium, found in diverse habitats, genetically accessible, frequently investigated for filament formation and nitrogen fixation, available omics data	[10,11,17,18, 36,37]
Tolypothrix sp.	PCC 7712	Heterocyst-forming filamentous cyanobacterium, food-grade phycocyanin production, applied in open pond systems, found in diverse habitats	[11,19]
Synechocystis sp.	PCC 6803	Model unicellular cyanobacterium, found in diverse habitats, available omics data, genetically accessible, previously applied for dual-species biofilm cultivation	[6,11,25,39]
Synechococcus elongatus	UTEX 2973	Model unicellular cyanobacterium, genetically accessible, fast autotrophic growth rate, tolerance to elevated temperatures and light intensities, available omics data	[21,40,41]
Leptolyngbya sp.	PCC 7104	Filamentous cyanobacterium, found in diverse habitats, high phycocyanin containing biomass production, tolerance to high temperatures and light intensities	[10,11,20]

shown that *Nostoc* sp. secretes high amounts of capsular/bound polysaccharides and released polysaccharides [12], both being important for EPS structure and thus attachment behavior.

Here, we evaluate different cyanobacterial strains including those described by Bharti et al. as being abundant in natural mats for their ability to form biofilms in capillary biofilm reactors (hereafter CBRs). A couple of unicellular growing cyanobacteria (*Synechocystis* sp., *Synechococcus* sp., *Cyanothece* sp.) were compared to filamentous ones (*Tolypothrix* sp., *Nostoc* sp., and *Leptolyngbya* sp.). Furthermore, biofilm formation in the CBR was investigated under nitrogen fixing conditions versus addition of nitrate to the medium. The CBR concept was introduced by Hoschek et al. as a means to cultivate cyanobacterial whole-cell biocatalysts at high cell-densities in a continuous mode, using the well-established cyanobacterial lab strain *Synechocystis* sp. PCC 6803 as model organism [6]. Furthermore, CBRs profit from an excellent surface to volume ratio of 1333–4000 $m^2\ m^{-3}$ and low light penetration depth preventing light limitation in the system and increasing the light to chemical energy conversion efficiency [5,6,13,14]. Here, we wanted to extend the CBR concept to other cyanobacterial species, as a first step towards establishing them as potential photo-biotech workhorses. As performance indicators biomass formation, surface coverage and biofilm stability have been monitored.

2. Materials and methods

2.1. Chemicals and media

Chemicals were of the highest purity available from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), or Carl-Roth GmbH (Karlsruhe, Germany). Standard media BG11-0 (lacking a nitrogen source, NaCl addition instead of $NaNO_3$) and BG11 were used for the cultivation of cyanobacteria [15] with addition of 10 mM HEPES (pH 7.4). LB complex media and M9 minimal media were used for the cultivation of *Pseudomonas taiwanensis* VLB120 and *Escherichia coli* [6], if not indicated otherwise.

2.2. Strains selected for this study

Six cyanobacteria species, whose main features are reported in Table 1, were selected for this study.

Unicellular *Cyanothece* sp. was selected due to its ability to fix atmospheric N_2 under diurnal conditions. Moreover, strain PCC 7822 is genetically accessible and is one of the species known for significant H_2 production [16]. *N. punctiforme* is one of the model organism frequently used for investigating filament formation and nitrogen fixation by forming heterocysts [17,18]. This strain is genetically accessible as is *Cyanothece* PCC7822. N_2 fixing filamentous *Tolypothrix* sp. is also a good candidate for biofilm research since it has been already used for outdoor biofilm cultivation and valuable food-grade phycocyanin production [19]. *Leptolyngbya* sp. is a species surviving in desert type environments and is highly suited for potential outdoor applications. It has been already used for production of high phycocyanin containing biomass at high temperatures (up to 40 °C) and light intensity (up to 1800 μmol $m^{-2}s^{-1}$) [20]. Additionally, *Synechococcus elongatus* UTEX 2973 was selected due to its ability to tolerate elevated temperatures (41 °C), light intensity of up to 500 μmol $m^{-2}s^{-1}$ and CO_2 (3%), and its exceptional high doubling time of 2.1 ± 0.2 h, which is the highest reported for a cyanobacterial species so far [21].

2.3. Cultivation and maintenance of microorganisms

Leptolyngbya PCC 7104, *Tolypothrix* PCC 7712, *Synechocystis* PCC 6803, and *Cyanothece* PCC 7822 were obtained from Pasteur Culture Collection (PCC); *Synechococcus elongatus* UTEX 2973 was obtained from the Culture Collection of Algae at The University of Texas (UTEX); *Nostoc punctiforme* ATCC29133 was obtained from the American Type Culture Collection (ATCC). Cyanobacterial strains were stored at a high cell density (10–15 days of growth before storage) in BG11 media supplemented with 7% DMSO at −80 °C.

All cyanobacteria species used in this study were revived by incubation on BG11 agar media at 25 μmol m^2s^{-1} LED illumination in a Multitron Pro incubator (Infors AG, Bottmingen, Switzerland). Subsequently, they were transferred to BG11 broth media containing 5 mM $NaHCO_3$ and incubated continuously under 50 μmol $m^{-2}s^{-1}$ illumination except for *Cyanothece* PCC 7822 which needed diurnal cycles of 16 h light:8 h dark. Cultivation conditions for each species have been summarized in Table 2.

Pseudomonas taiwanensis VLB120 and *Escherichia coli* W3110 were revived on LB agar media. Subsequently, they were transferred to LB broth media and later into M9 minimal media prior to mixing with the respective cyanobacteria. They were always grown with respective supplements at standard conditions (Table 2) in an Ecotron incubator (Infors AG, Bottmingen, Switzerland).

2.4. Capillary biofilm reactor setup

Fig. 1 shows a schematic representation of the CBR system.

The CBR system was constructed as previously described in Ref. [6] with slight modifications. Custom-made Polymethylmethacrylate (PMMA) capillaries with 3 mm inner diameter were cut to 20 cm length and used as cultivation devices. Capillaries and tygon tubes were connected by using polypropylene connecters (Ismatec, Wertheim, Germany). T-connecters were used for simultaneous supply of air and media to the system via a peristatic pump (Ismatec, Wertheim, Germany), which was adjusted to a flow rate of 52 μl min^{-1}. A LED light system (450 W regelbare Wachstumslampe, CellDEG, Germany) was installed on top of the capillaries to supply light at 50–60 μmol $m^{-2}s^{-1}$. Injection ports were attached directly in front of capillaries, bubble traps and

Table 2
Standard growth conditions applied for the species used in this study.

Species	Temperature, rotating	Light: Dark	Media	Supplements
Cyanothece sp. PCC 7822	30 °C, 120 rpm	16 h: 8 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
			$BG11_0$	
Nostoc punctiforme ATCC29133	30 °C, 150 rpm	24 h: 0 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
			$BG11_0$	
Tolypothrix sp. PCC 7712	30 °C, 0 rpm	24 h: 0 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
			$BG11_0$	
Synechocystis sp. PCC 6803	30 °C, 150 rpm	24 h: 0 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
Synechococcus elongatus UTEX 2973	30 °C, 150 rpm	24 h: 0 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
Leptolyngbya sp. PCC 7104	30 °C, 0 rpm	24 h: 0 h	BG11	5 mM $NaHCO_3$, ambient air, 75% humidity
Pseudomonas taiwanensis VLB120	30 °C, 200 rpm	–	LB	30 μg/ml gentamycin
			M9	30 μg/ml gentamycin, US* trace elements, 5 g/L glucose
Escherichia coli W3110	30 °C, 200 rpm	–	LB	
			M9	US* Trace elements,0.01% thiamine, 5 g/L glucose

sampling ports were implemented behind the capillaries.

2.5. CBR operating conditions

2.5.1. Cyanobacteria cultivation

Standard cultivation conditions for each strain are listed in Table 2. In brief, cyanobacteria pre-cultures were grown under standard conditions in respective media (Table 2) for one week. Subsequently, they were transferred into fresh BG11 or BG11-0 media and after 7 days of incubation under standard conditions, 1 ml of each culture was centrifuged and the biomass was determined via chlorophyll *a* (Chl *a*) quantification (see below 2.6).

2.5.2. Pseudomonas taiwanensis VLB120 and Escherichia coli W3110 cultivation

Heterotrophic bacterial pre-cultures were grown overnight under standard conditions (Table 2) in LB media. Subsequently, 200 μl were transferred to 20 ml M9 minimal media with respective supplements (Table 2). After overnight growth under standard conditions, 200 μl cell culture were transferred again to fresh M9 media with same supplements. After 7–8 h incubation, optical density was measured at 450 nm by Libra S11 visible spectrophotometer (Biochrom, Cambridge, UK).

2.5.3. Inoculum preparation

In case of dual species cultivation *Pseudomonas* sp. cultures were washed twice with respective cyanobacteria media in order to avoid antibiotic contamination and then re-suspended in 5 ml of the same media to an OD_{450} value of 2. Cyanobacteria cultures were centrifuged and re-suspended in 5 ml of respective cyanobacteria media to a Chl *a* concentration of 16 μM. Finally, both were mixed (5 ml of cyanobacteria culture (16 μM Chl *a*) and 5 ml of *Pseudomonas* culture (OD_{450} of 2)) obtaining a final Chl *a* [μM]: OD_{450} ratio of 8:1 in the resulting 10 ml of culture volume. This mixed culture was grown overnight under the standard conditions of the respective cyanobacterial species (Table 2). Same protocol was applied for the mixing of *E. coli* W3110 with cyanobacterial species. No organic carbon to support growth of heterotrophic organisms was added to the mixed-trophies cultures.

2.5.4. Inoculation and cultivation

Inoculation of the CBR was conducted by injection of 2.5 ml mixed culture through the injection port located in front of the capillaries. After inoculation, capillaries were covered with aluminum foil and kept idle overnight before the medium flow was set to 52 μl min^{-1} and the illumination was set to constant 50–60 μmol $m^{-2}s^{-1}$. After 4 days of biofilm growth the air feed supplying ambient air was started by adjusting the same flow rate as for the media feed. In case of *Cyanothece* PCC 7822, diurnal cycles were applied as stated in Table 2. All experiments were conducted in biological duplicates and they were all incubated at room temperature for 21 days.

2.6. Analytical methods

O_2 concentration was measured in the air phase in bubble traps via a PyroScience O_2 optode (PyroScience GmbH, Aachen, Germany), a sensor system which concomitantly recorded the O_2 concentration and temperature. Dissolved O_2 concentration in the aqueous phase was calculated based on Henry's law.

H_2 concentration was measured on a TRACE 1310 gas chromatograph (Thermo Scientific). The device was equipped with a 30 m long TracePLOT TG-Bond MSieve 5A column which had 0.32 mm inner diameter and 0.20 μm film thickness (Thermo Scientific). Temperature of the thermal conductivity detector and the oven were adjusted to 100 °C and

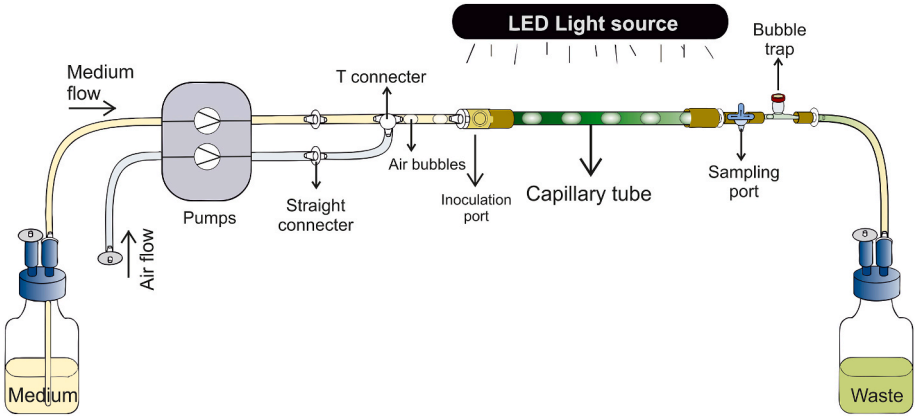

Fig. 1. Schematic representation of the capillary biofilm reactor (CBR) used in this study.

75 °C, respectively. 100 μl of sample volume was injected manually after collecting the sample from the bubble trap. The flow rate of the carrier gas (argon) was set to 2 mL min^{-1}, with a total running time of 2 min. Defined gas mixtures of H_2 (0.01%, 0.1%, 1%, 2%, 10%) were used to set the calibration curve and H_2 concentration was determined from the curve equation.

Biofilm biomass determination: Biofilm samples were collected after 21 days by manually scratching the biomass out of the capillaries using a needle with a syringe and re-suspend it in 20 ml BG11 media. IKA Ultra Turrax Tube Disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany) was used at max speed for 5 s in order to homogenize samples. 10 mL of the homogenized suspension was filtered through pre-weighed 0.2 μm Whatmann filter made out of regenerated cellulose membranes (50 mm diameter, GE Healthcare Life Sciences, Chicago, US). Filters were dried at 80 °C for 3 days (Binder incubator, BINDER GmbH, Tuttlingen, Germany) and then transferred into a desiccator before measuring weight. All measurements involved biological replicates originating from the capillaries.

Chl a and carotenoid measurements were performed as described in Ref. [22]. Pre-cooled 1 ml methanol (≥99.9%, HPLC grade, The Carl Roth GmbH, Karlsruhe, Germany) was used to dissolve the biomass obtained by centrifuging 0.5–1 ml of biofilm cultures. Samples were homogenized by vortexing (Vortex Genie 2, Scientific Industries Inc., New York, US), wrapped in aluminum foil and incubated at 4 °C for 20 min. After centrifugation of the samples, absorbance of the supernatant was measured at 470 nm, 665 nm, and 720 nm (Cary 300 UV–Vis spectrophotometer; Agilent Technologies, Santa Clara, US) to calculate Chl *a* content (Eq. (1) & Eq. (2)). Besides biological duplicates, technical duplicates were added for pigment measurements. For planktonically growing cultures (section 2.3; Table 2) the full absorbance spectrum (375 nm–800 nm) was measured after methanol extraction [22].

$$\text{Chl a}\,[\mu g/ml] = 12.9447 \times (A665 - A720) \quad \text{(Eq.1)}$$

$$\text{Chl a}\ [\mu M] = 14.4892 \times (A665 - A720); \quad \textit{for}\ \text{Chl a MW} = 893.4890\ g/mol \quad \text{(Eq.2)}$$

Chemical oxygen demand (COD) was determined using a LCK614 kit (50–300 mgL^{-1} O_2 range, HACH, Loveland, US). At day 21, 10 mL were collected from capillaries' outflow. 5 mL were filtered through a 0.2 μm Whatman filter, while 5 mL were used directly without filtration for COD and pH-measurement. Samples were kept at −20 °C until COD analyses. COD of filtered (soluble COD; sCOD) and non-filtered (total COD) outflow samples was determined according to manufacturer's protocol. Briefly, 2 mL of diluted samples were added to the pre-mixed test tubes and incubated at 170 °C for 15 min inside a HT200S High Temperature Thermostat (HACH, Loveland, US). After cooling down to room temperature, samples' COD was measured using a DR1900 Portable VIS spectrophotometer (HACH, Loveland, US). Particulate COD (pCOD) was calculated by subtracting soCOD value from total COD value.

2.7. EPS staining

Cyanobacterial planktonic cultures (Table 1), grown for 1 week, were centrifuged at 5000 g for 10 min. 1–2 drops of Alcian blue pH 2.5 (stains most acid mucins, except some of the strongly sulfated groups) (Merck KGaA, Darmstadt, Germany) were placed on microscope glass slides and cells scratched from the pellet directly mixed into Alcian blue droplets with an inoculation loop. After air-drying for 20–30 min, samples were gently washed with tap water and 1–2 drops of safarinO were added on microscope slides. Samples were again air-dried for 20–30 min and then gently washed with 95% EtOH. The residual EtOH solution was dried and the respective standard medium (Table 2) added prior analysis with Light Microscopy (LM) (ZEISS, Oberkochen, Germany). The same staining procedure was followed with Alcian blue pH 1 (stains strongly sulfated acid mucins).

2.8. Surface coverage analysis using ImageJ

During biofilm cultivation, images from the capillaries were collected daily to document biofilm growth. Images were transferred to ImageJ v.1.45s (https://imagej.nih.gov/ij/) and converted to 8-bit. After scaling based on capillary length, surface areas of the capillaries were selected and analyzed by the 'Plot profile' feature. Background noise was obtained by selecting and plotting the regions next to the biofilms. Raw data of all plotted images were extracted as CVS file and assigned to Origin 2019 (OriginLab corp., Northampton, US) for further calculation. Surface coverage was determined subtracting all the values obtained from the plot profile of the sample images from the background noise constant and integrating along the capillary length.

2.9. Statistical analyses

Microsoft Excel 2013 (Microsoft, Redmond, US) and Origin 2019 (OriginLab corporation, Northampton, US) were used to compute statistical parameters. Capillaries were set up as biological duplicates and each of them contained at least 2 technical replicates for every measurement except dry biomass measurement which only had biological replicates.

3. Results

All selected species (Table 1) were cultivated in suspension as well as in a CBR (Fig. 1), which has recently been reported to achieve high cell densities in the case of *Synechocystis* sp. PCC 6803 [6]. Unicellular cyanobacteria were compared to filamentous ones, as well as biofilm formation under nitrogen fixing conditions versus biofilm formation in the presence of nitrate. Important for achieving such high cell densities was the co-cultivation of *Synechocystis* sp. PCC 6803 together with the well described biofilm former *P. taiwanensis* VLB120 [6]. Thus, in this study, different combinations of strains (mixed, single species, *P. taiwanensis* or *E. coli* W3110 as supporter strain) were investigated, focusing on biomass formation and surface coverage of the available growth surface. Biofilm dry weight (BDW) and chlorophyll *a* (Chl *a*) concentrations were used to determine final biofilm biomass. ImageJ analyses were conducted to gain numerical data visualizing biofilm development. Planktonic and/or detached biomass in the bulk phase was quantified by determining the COD of outflow samples.

3.1. Initial microscopic analysis of the candidate strains

All strains in this study were submitted to a careful microscopic analysis (Fig. 2), with a special focus on possible EPS production, which is a major component of the biofilm matrix [7]. Alcian blue and SafarinO were applied to distinguish EPS and cells. Using Alcian blue pH 1.0 and pH 2.5 possible sulfated regions could be identified as well. If applicable, nitrogen fixing conditions have been compared to non-fixing conditions.

Fig. 2 reveals distinct differences in EPS amount and the organization of the cells within, depending on the culture conditions, especially for *Nostoc* sp., which produced more and distinct EPS under nitrate replete condition compared to nitrogen fixing conditions. Also for *Tolypothrix* sp. a significant amount of tubular EPS around the filaments was observed under both cultivation conditions. Alcian pH 1.0 staining revealed a high amount of sulfated EPS. *Leptolyngbya* sp. PCC 7104 displayed similar EPS formation to *Tolypothrix* sp. as surrounding filaments in a tubular form. The highest EPS amount was determined for *Cyanothece* sp., which appeared as kind of cell-EPS flocs independent of the respective culture condition. This resembles 'cyanoflan' a recently reported sulfated carbohydrate polymer with emulsifying properties produced by the marine cyanobacterium *Cyanothece* CCY 0110 [23]. In contrast, no or very little EPS could be detected for *S. elongatus* and *Synechocystis* sp., respectively, under the cultivation conditions and the respective staining protocols applied.

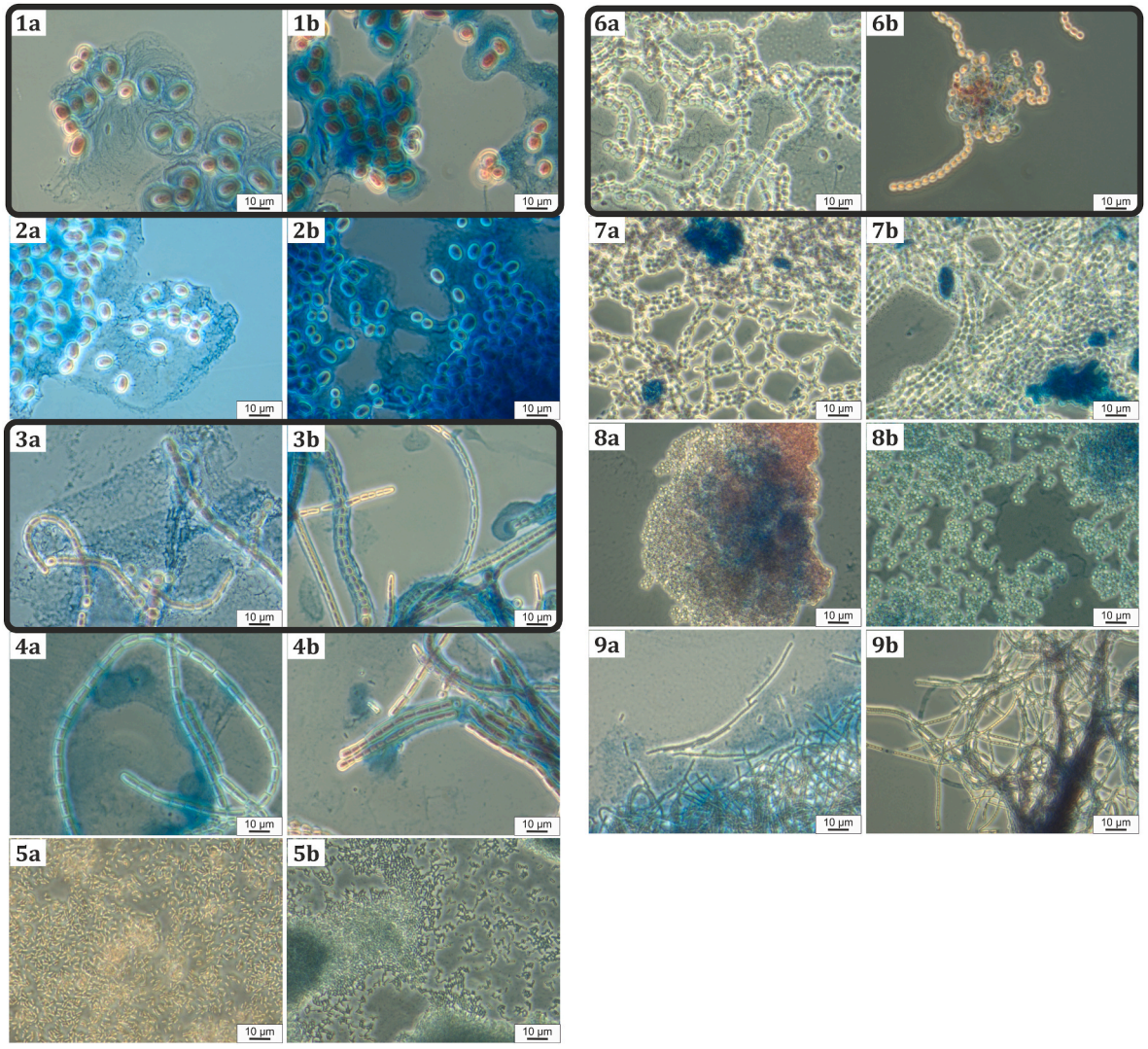

Fig. 2. Microscopy analysis of the candidate strains listed in Table 1. Staining was achieved via Alcian blue pH 1.0 **(a)** for the sulfated regions of EPS and pH 2.5 **(b)** for the general EPS fraction. SafarinO was used as counterstain for the cells. All specimen originated from batch axenic cyanobacterial cultures. If applicable, cultures were cultivated under nitrogen fixing conditions (images covered with a black frame) and in the presence of $NaNO_3$ (images without a frame). **1**–**2** *Cyanothece* sp. PCC 7822; **3**–**4** *Tolypothrix* sp. PCC 7712; **5** *S. elongatus* UTEX 2973; **6**–**7** *N. punctiforme* ATCC29133; **8** *Synechocystis* sp. PCC 6803; **9** *Leptolyngbya* sp. PCC 7104.

3.2. The importance of *P. taiwanensis* as biofilm supporter strain

In previous works conducted with *Synechocystis* sp. PCC 6803, it was shown that the addition of the chemoorgano-heterotrophic *P. taiwanensis* VLB120 positively impacted biofilm formation of *Synechocystis* in the CBR [6]. The nature of this interaction which leads to a dense, high biomass biofilm in the CBR is still a matter of investigation. Here, we conducted a preliminary experiment concerning single- and dual-species (coupled with *P. taiwanensis* VLB120) CBR experiments with other two cyanobacterial species *Tolypothrix* sp. PCC 7712 and *Leptolyngbya* sp. PCC 7104 (Supplementary data Fig. S1). They both showed significantly higher (3–4 fold) biomass formation in the case of dual-species compared to single-species CBRs.

In addition, *E. coli* other was investigated as a biofilm supporter organism for the cyanobacteria in the CBR. *E. coli* W3110 was chosen, due to its known biofilm forming ability [24]. *Synechocystis* sp. PCC 6803 and *Tolypothrix* sp. PCC 7712 were co-cultivated with *E. coli* W3110 as described in the materials and method section.

Fig. 3 shows, that both cyanobacterial strains developed more biomass and a higher Chl *a* concentration when co-cultivated with *P. taiwanensis* VLB120. This effect is more pronounced for *Synechocystis* sp., which produced approximately 6 times more biomass in the presence of *P. taiwanensis* as compared to *E. coli*. In the case of *Tolypothrix* sp., the two supporter strains resulted in similar biomass formation and surface coverage, although values were slightly higher for the co-culture containing *P. taiwanensis*. This might be due to the filamentous structure of *Tolypothrix* sp. and the higher EPS production, which facilitates surface attachment. As co-cultivation with *P. taiwanensis* leads to higher biomass formation, further experiments, aimed at comparing various cyanobacterial species for their biofilm formation ability, were conducted using *P. taiwanensis* as supporter microorganism in the dual-species CBR cultivations.

3.3. Biomass development in the CBR

All candidate strains were cultivated in the CBR as mixed cultures containing the respective cyanobacterial strain and *P. taiwanensis* as biofilm supporter strain. To prevent carbon limitation, the cultivation medium was supplemented with 5 mM $NaHCO_3$. Surface coverage, final biomass indicated by Chl *a* content and biomass dry weight, as well as the detached biomass (COD measurements) were monitored. Furthermore, biofilm formation under nitrogen fixing conditions and in the

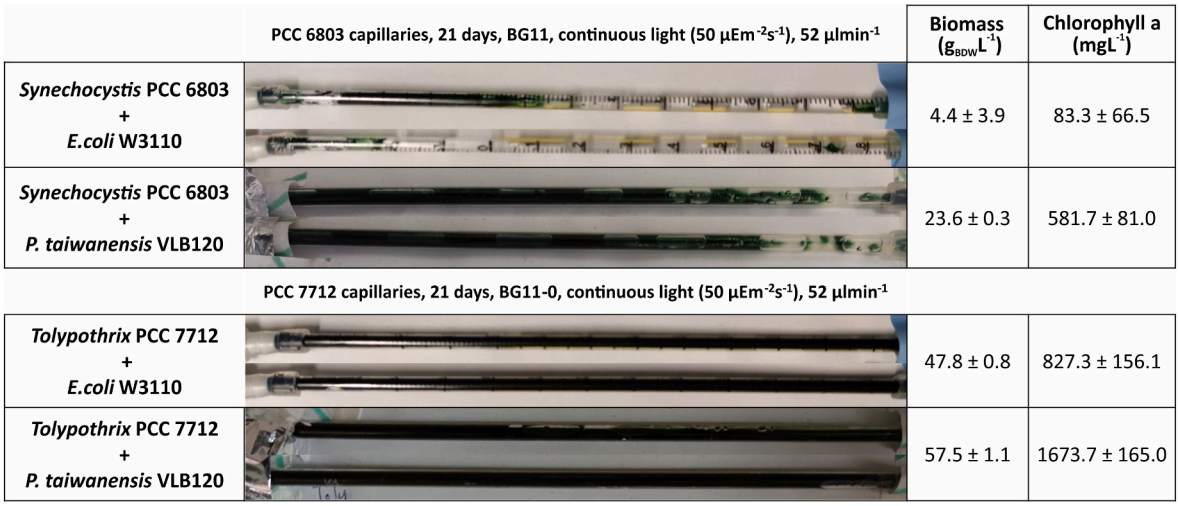

Fig. 3. Images of CBRs showing the difference between *E. coli* and *P. taiwanensis* as biofilm supporter strains in dual-species phototrophic biofilms.

presence of nitrate was investigated where applicable.

While all filamentous cyanobacteria in the study showed considerable biofilm formation, the unicellular species *Cyanothece* sp. and *Synechococcus* sp. barely attached to the respective surface (Fig. 4). The model organism *Synechocystis* sp. seemed to be an exception here, as it was the only unicellular strain growing in the CBR. *S. elongatus* did not produce notable amounts of EPS (Fig. 2), which might have negatively impacted its attachment behavior.

The highest biomass formation and Chl *a* content under all cultivation conditions tested were determined for *Tolypothrix* sp. cultures, which produced 62.6 ± 6.3 and 57.5 ± 1.1 $g_{BDW}L^{-1}$ biomass in BG11 and BG11-0 media, respectively. It showed almost 3-fold higher biomass production in terms of BDW L^{-1} and Chl *a* content compared to *Synechocystis* PCC 6803. All other candidates showed significantly less biomass production. Surprisingly, *N. punctiforme* ATCC 29133 produced more biomass under nitrogen fixing conditions. This observation correlates with microscopic analysis (Fig. 2), which revealed that the process of nitrogen fixation seemed to impact EPS formation in the case of *N. punctiforme* and *Tolypothrix* sp. However, a high yield on EPS does not necessarily lead to more biofilm in the CBR, as indicated by *Nostoc* sp. data, showing lower biomass under nitrate replete condition even though more EPS was formed. Furthermore, *Cyanothece* sp., which secreted considerable amounts of EPS did not attach at all in our system. *Leptolyngbya* PCC 7104 also produced more biomass (28.1 ± 13.5 $g_{BDW}L^{-1}$) compared to *Synechocystis* PCC 6803, however, the standard deviations were very high. This was due to one of the biological replicates partly detaching 5 days after inoculation. Although the biofilm slowly recovered again, this sloughing event heavily impacted the standard deviation of all subsequent measurements.

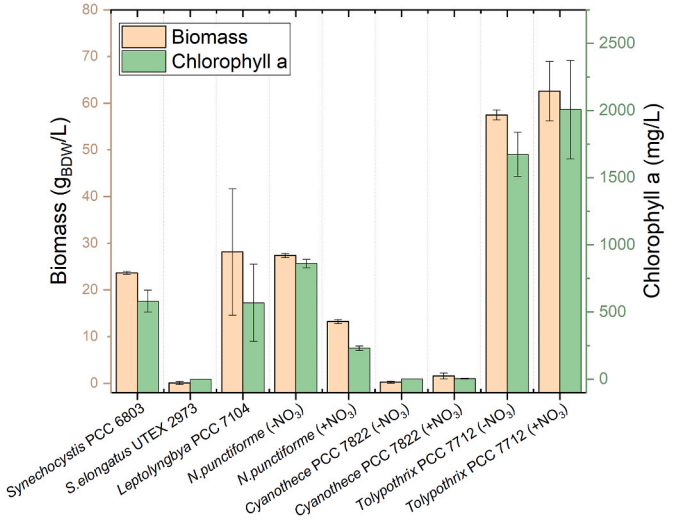

Fig. 4. Biomass and Chl *a* concentration derived from co-culture CBR cultivations, each coupled to the heterotrophic partner *P. taiwanensis* VLB120, over 21 days. Each experiment was conducted as biological duplicate.

For all strains the bleed stream leaving the CBR was monitored to estimate how efficient the biomass attached inside the reactor. Here, we determined the COD (mgL^{-1}) of sCOD and non-filtered outflow samples followed by subtracting these values to achieve the pCOD. Finally, the values were normalized to the final biomass ($g_{BDW}L^{-1}$) obtained after 21 days of CBR operation (Table 3).

The carbon loss due to pCOD was determined to be 0.49 ± 0.11 and 0.91 ± 0.26 mg_{pCOD}:g_{BDW} for *Tolypothrix* sp. growing either in nitrate-deficient or nitrate replete media, respectively, which are the lowest values of all species tested. Similarly, the sCOD were low in this culture, calculated as 0.77 ± 0.28 and 1.82 ± 0.38 mg_{sCOD}:g_{BDW} for nitrate-deficient and nitrate repleted media, respectively (Table 3). Even though *N. punctiforme* showed a low detachment rate when being cultivated under nitrogen fixation conditions, the amount of final biomass in the capillaries was more than 2-fold higher for *Tolypothrix* sp. (Fig. 4). However, when nitrate was added to the medium, the COD values increased for both cultures but more dramatically for *N. punctiforme*.

As final parameter, the growth rate derived from the overall surface coverage of the different species was evaluated. In nearly all cases the biofilm started growing after the segmented flow was started at day four (Fig. 5).

Table 3
Carbon loss via the outflow of the CBR under different cultivation conditions given in mg_{COD} per g_{BDW} (sCOD: soluble COD; pCOD: particulate COD; 0.20 µm cutoff).

Capillaries	Nitrogen source	mg_{sCOD}: g_{BDW}	mg_{pCOD}: g_{BDW}
Synechocystis sp. PCC 6803	$NaNO_3$	2.28 ± 0.93	2.22 ± 0.24
S. elongatus UTEX 2973	$NaNO_3$	ND	ND
Leptolyngbya sp. PCC 7104	$NaNO_3$	3.45 ± 0.83	2.95 ± 2.30
N. punctiforme ATCC29133	N_2	1.46 ± 0.65	0.92 ± 0.27
N. punctiforme ATCC29133	$NaNO_3$	7.52 ± 2.49	33.9 ± 5.36
Cyanothece sp. PCC 7822	N_2	ND	ND
Cyanothece sp. PCC 7822	$NaNO_3$	ND	ND
Tolypothrix sp. PCC 7712	N_2	0.77 ± 0.28	0.49 ± 0.11
Tolypothrix sp. PCC 7712	$NaNO_3$	1.82 ± 0.38	0.91 ± 0.26

Synechocystis sp. grew slowly in a linear fashion after segmented flow was started at day 4, whereas *Tolypothrix* sp. biofilms grew comparably fast as indicated by the surface coverage data. Especially after the start of segmented flow on day 4, surface coverage increased significantly until day 11 for capillaries fed with $NaNO_3$. In nitrogen fixation conditions, surface coverage by *Tolypothrix* sp. biofilms started to expand strongly even before segmented flow was started, the only culture where such a behavior could be observed. This difference in the initial growth phase (day 0–4) might have been caused by increased EPS production under nitrogen fixing conditions, which, again, might have been triggered by the need to ensure an oxygen free environment around the heterocysts. Furthermore, *Tolypothrix* sp. is the only strain clearly reaching a steady state during the 21 days cultivation time. The *Nostoc* sp. strain investigated in this study, which is also fixing nitrogen via heterocysts, initially showed a comparable behavior. When fixing atmospheric nitrogen, it appeared as if the biofilm directly started to grow, as indicated by the rising surface coverage. However, upon the segments' start, huge parts of *Nostoc*-biofilm were detached before a linear growth was reestablished, thus the attachment forces at this stage were clearly different between these two species.

pH was monitored in addition to surface coverage. As expected, pH increased in every capillary whenever high biomass was observed (Fig. 5) due to photosynthetic activity and carbon uptake by organisms, which has been described in detail for *Synechocystis* sp. [25]. *Synechocystis* sp. takes up the HCO^{3-} via a Na^{+}/HCO^{3-} symporter and concomitantly imports protons via an Na^{+}/H^{+} antiporter to balance the inner pH of the cell and thus causes a pH increase in the extracellular environment [25].

Overall, this study identified a novel candidate to be applied as biocatalyst in CBRs (Table 4). *Tolypothrix* PCC 7712 grew to high cell densities, showed a strong surface attachment and low carbon loss.

3.4. Continuous hydrogen production by Tolypothrix PCC 7712 biofilms in a CBR

To check the suitability of the CBR system operated with *Tolypothrix* sp. PCC 7712 for a possible application, the hydrogen evolution during nitrogen fixation has been investigated (Fig. 6).

Fig. 6 shows the constant H_2 evolution of *Tolypothrix* sp. PCC 7712 biofilm coupled with *P. taiwanensis* VLB120 during nitrogen fixation. Remarkably, the reaction is constant over a time period of 14 days, before the experiment was actively terminated. H_2 production reached

Table 4
All the parameters related to the biofilm formation investigated in this study are qualitatively summarized in the table.

Capillaries	Nitrogen source	EPS formation	Surface coverage	*Biomass/ Chl* a	mg_{pCOD}/g_{BDW}
Synechocystis sp. PCC 6803	$NaNO_3$	+-	+	++	++
S. elongatus UTEX 2973	$NaNO_3$	–	–	–	–
Leptolyngbya sp. PCC 7104	$NaNO_3$	++	+	++	++
N. punctiforme ATCC29133	N_2	+	++	++	+++
N. punctiforme ATCC29133	$NaNO_3$	++	++	+	+
Cyanothece sp. PCC 7822	N_2	+++	+-	–	–
Cyanothece sp. PCC 7822	$NaNO_3$	+++	+-	–	–
Tolypothrix sp. PCC 7712	N_2	+++	+++	+++++	+++
Tolypothrix sp. PCC 7712	$NaNO_3$	+++	+++	+++++	+++

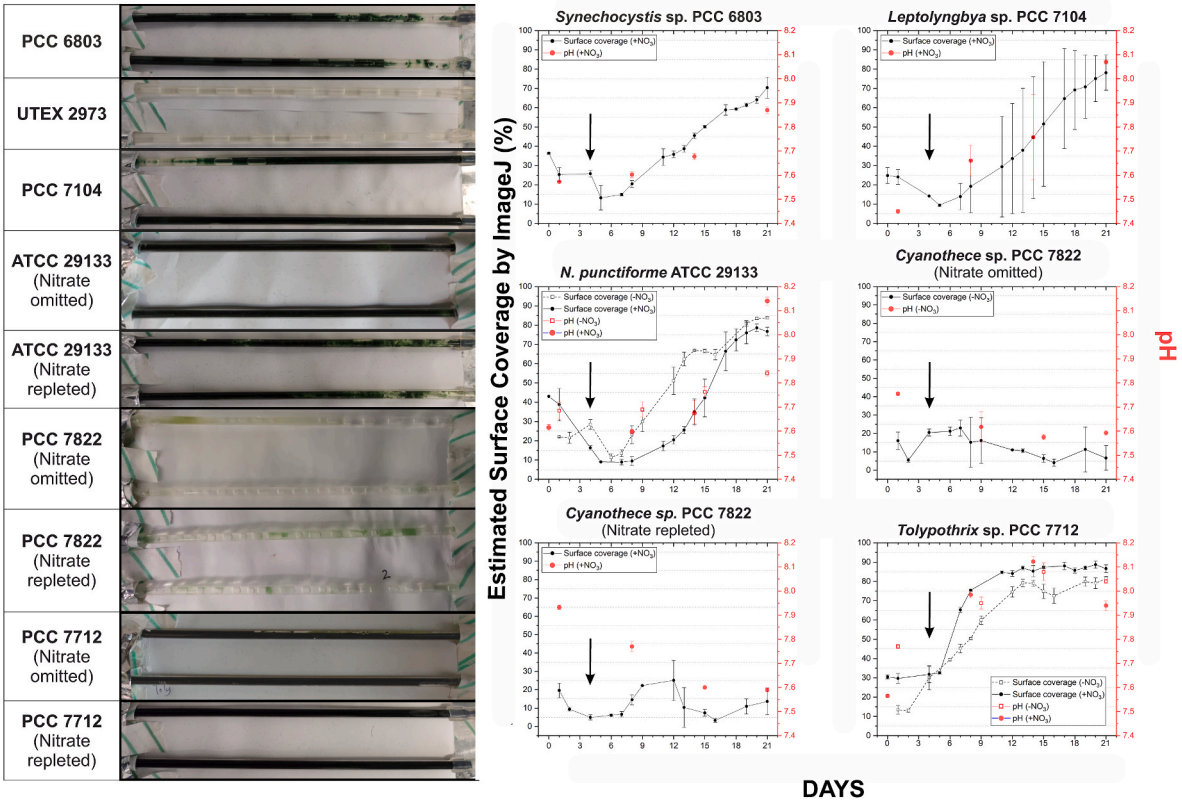

Fig. 5. Surface coverage and pH development of all strains investigated in CBRs over a cultivation time of 21 days calculated by ImageJ. Arrows on the graphs indicates day 4 when segmented flow was started. Images on the left side show the state of the CBR on day 21. All cultivations were conducted with *P. taiwanensis* VLB120 as biofilm supporter strain.

1.23 mmol L^{-1} and a H_2 production rate of 0.14 μmol H_2 $mg_{Chl\ a}^{-1}h^{-1}$.

4. Discussion

In this study we wanted to extent the application of the CBR, which was so far only operated with *Synechocystis* sp. PCC 6803, to other cyanobacteria. The CBR technology was shown to enable high cell densities and continuous bioprocessing, making it a promising system for productive whole-cell catalysis. However, the system is restricted to approaches, where cells are functioning as solar cell factories, meaning that they constantly excrete a product of choice which can easily be extracted from the broth. It is not suited for biomass based products. Scaling is possible via numbering up, as shown for other microscale production systems [13]. Despite their large presence in microbial mats, growing cyanobacteria in a technical environment may turn out to be sophisticated, depending on the bioreactor applied. A crucial parameter is the O_2 concentration in the system. Due to oxygenic photosynthesis by cyanobacteria, O_2 may accumulate in the bioreactor, reaching high concentration that promote the formation of radical oxygen species (ROS), which finally lead to cell toxification [26]. Running the CBR in a segmented flow fashion (with air segments) facilitates oxygen removal from the system, keeping it at around 250 μmol L^{-1} [6]. Furthermore, the co-cultivation with an O_2 respiring organism like *P. taiwanensis* VLB 120 is beneficial in this aspect.

The EPS, as an essential part of a biofilm, may be a good indicator if the organisms are prone to attach tightly to a surface. Cyanobacteria are well known to produce EPS, also, if they are growing planktonic. Cyanobacterial EPS can stay fixed to the cell surface, either as capsule, slime form, or sheath, or be secreted into the proximate environment. Furthermore, EPS exhibit extreme structural complexity as polymers, which makes them a very promising product in biotechnological applications [23]. Moreover, various cyanobacteria are known to produce EPS which favors the binding of metal ions [27]. Sometimes, the excretion of EPS is part of the cellular stress response system, like described for *Synechocystis* sp. [28]. In a biotechnologically employed system, the EPS is a double-edged sword. On the one side, it is needed for cell attachment and protection. On the other side it represents a substantial mass transfer barrier, hampering the transfer of compounds in and out of the cells. Furthermore, resources in terms of carbon, nitrogen and electrons are lost if they end up in the EPS. In an ideal biocatalytic system, the major portion of available electrons and carbon is channeled into the product of choice, while only a minor amount is utilized for biomass generation and maintenance. In reality, microbial systems use a large fraction of the available resources for biomass, soluble microbial products (SMPs), and EPS production [29]. For *Synechocystis* sp. PCC 6803 growing in batch cultures it was shown that up to 84% of the available resources are used for biomass formation, whereas up to 25% end up in SMPs. A strain modified to produce lauric acid channeled up to 30% into SMP production [30]. For biofilms, such investigations are missing so far. Our data show that also in attached growth modes, significant amounts of SMPs are produced and are constantly flushed out of the system, together with detached cells and EPS compounds. In addition, cyanobacterial biofilms are prone to encounter 'catastrophic sloughing events' during which large parts of the biofilm detach, like observed for *Leptolyngbia* sp. in our study. These events are not well understood. Presumably, it is a mechanism to support biofilm regeneration and thus overall system robustness. However, from an economic perspective, it means that large amounts of the biocatalyst are lost, together with a significant portion of the nutrients, carbon and electrons. Together with the SMPs, sloughed cells may account for 50% of the total carbon and energy fixed during photosynthesis. To establish an economic process based on biofilms, it will be important to minimize such losses and think about measures to reuse these waste products.

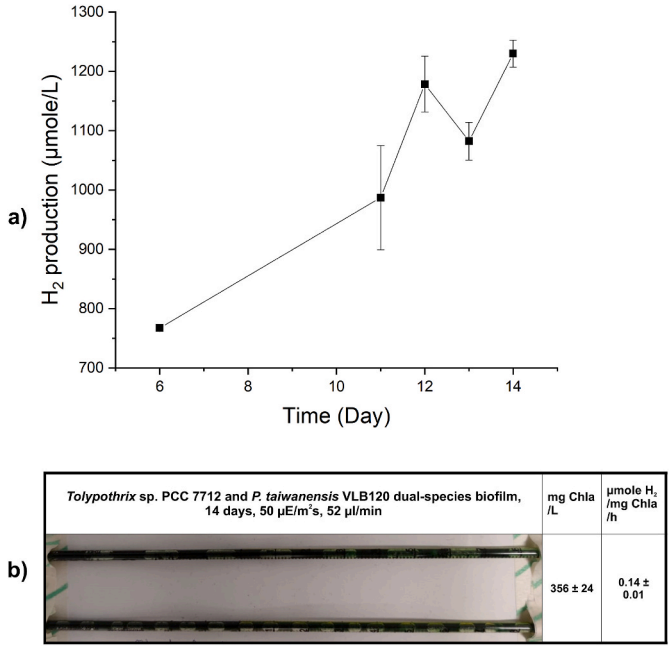

Fig. 6. Continuous production of H_2 in a CBR containing a mixed trophies biofilm of *Tolypothrix* sp. PCC 7712 and *P. taiwanensis* VLB120 under N_2-fixing conditions. Daily H_2 production **(a)** and image of the respective CBR with the corresponding Chl a concentration and H_2 production rate at day 14 **(b).**

In our study *Tolypothrix* sp. and *N. punctiforme* both showed very low carbon loss during nitrogen fixation. Their filamentous morphology might promote the attachment efficiency by creating a very effective 3-D structure. Since they are both heterocyst-forming filamentous cyanobacteria, the low detachment values could also be attributed to heterocyst formation, which may cause a stronger attachment on the capillary surface. It is known that the envelope of heterocysts is a thick structure consisting of specialized glycolipids and polysaccharides that shields the nitrogenase from oxygen [31,32]. These additional EPS layers might be the reason behind less detachment occurring in nitrogen fixing conditions for *Tolypothrix* sp. and *N. punctiforme*.

This study showed, that we are only about to tap the huge potential of cyanobacterial workhorses and that there are many more highly interesting strains waiting to be discovered. *Tolypothrix* sp. PCC 7712 is self-sustaining in terms of carbon and nitrogen supply, which both can be utilized directly from the atmosphere. Thus, this strain is widely independent from expensive media components that have a significant carbon footprint in terms of nitrate supply. The results of this study suggest that *Tolypothrix* sp. PCC 7712 had a better performance compared to the current workhorse *Synechocystis* sp. PCC 6803, which cannot fix atmospheric nitrogen and, showed higher cell detachment and carbon loss, and less biofilm biomass formation in the CBR. *Tolypothrix* sp. has been already used for outdoor biofilm cultivation and valuable food-grade phycocyanin production [19]. Velu et al. [19] observed considerably high phycocyanin and biomass yields and very high metal removal rates while high CO_2 was supplied (15%) under meso-scale outdoor cultivation and nitrogen-fixing conditions. Additionally, hydrogen may be produced as a side product of the nitrogenase activity [33]. In our study, the hydrogen evolution rates are rather low. However, the CBR was not yet optimized for H_2 production and probably a significant fraction of the product was escaping from the reactor. Further, we could identify the uptake hydrogenase subunits HupS and HupL encoded on the genome of *Tolypothrix* sp. PCC 7712 (Unpublished results), which may also contribute to the low H_2 production [34]. Perspectively, deletion of these genes may improve the H_2 evolution, like shown for *Nostoc* sp. PCC 7422. In this strain it was possible to increase its H_2 production 3 fold by deleting the *hupL* gene [35].

5. Conclusion

This study provides a comprehensive assessment of biofilm formation of a number of cyanobacteria as defined-consortia in a CBR. Although the current study was limited to six species, it was evidenced, that there are huge differences between the tested strains, and that a couple of those performed superior compared to the current, broadly established workhorse *Synechocystis* sp. PCC 6803. Our findings indicate that *Tolypothrix* sp. PCC 7712 is a species with great potential in photobiotechnology. This is reflected in its high biomass production up to 62.6 $\pm$ 6.34 and 57.5 $\pm$ 1.08 $g_{BDW}L^{-1}$ in the nitrate-enriched and nitrate-deficient conditions, respectively. Furthermore, it needs 10 times less of $NaHCO_3$ compared to a previous study conducted by Hoschek et al. [6]. The continuous production rate of 0.14 μmole H_2 $mg_{Chl\ a}{}^{-1}h^{-1}$ of the wild type organism is a promising preliminary result for its future application for white H_2 production. These findings highlight the potential usefulness of other cyanobacteria besides the well-established species *Synechocystis* sp. PCC 6803.

Authorship

All authors have made substantial contributions to all of the following:

MB & KB: conception and design of the study
MB: acquisition of data
All: analysis and interpretation of data
MB & KB drafting the article or revising it critically for important intellectual content
All: final approval of the version to be submitted

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2022.100073.

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