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Comparison of ethanol tolerance between potential cyanobacterial production hosts

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ABSTRACT

Cyanobacteria are photosynthetic prokaryotes that have been extensively studied as potential autotrophic biotechnological hosts for the production of different carbon-based end-products directly from atmospheric CO_2. While commercially competitive applications do not yet exist, the production of ethanol in cyanobacteria is the most mature technology, endorsed by relatively high production yields and established status of ethanol in the global biofuel market. Within this concept, the aim here was to systematically compare ethanol tolerance of different commonly used cyanobacterial strains and substrains, in order to assess their relative potential for biotechnological production platforms. The comparison revealed clear strain-specific differences in ethanol toxicity, with growth inhibition GI_{50} values ranging between 3 g L^{-1} (0.4% V/V) and 28 g L^{-1} (3.5% V/V). The most tolerant wild-type strains were *Synechocystis* sp. PCC 6803 (substrain A) and *Synechococcus* sp. PCC 7002, which did not show any apparent effect in growth below ethanol concentrations 9.2 g L^{-1} (1.2% V/V). In comparison to typical biotechnological yeast strains used for ethanol fermentation, these values are clearly lower but still around the same order of magnitude. The results also underlined the challenges in direct number-based comparison between cyanobacterial strains and culture conditions due to inconsistencies in respect to chlorophyll content, cell morphology and optical properties.

1. Introduction

There is an increasing global need to find sustainable alternatives to replace fossil-based transport fuels in response to rising demand, environmental concerns related to elevating atmospheric CO_2 levels, and concurrent need to reduce greenhouse gas emissions. The current strategies for producing renewable fuels rely exclusively on the use of biomass-derived raw materials, but due to the enormous scale of the fuel-based economy, this is not sufficient to meet the global demand. This has prompted scientific interest towards parallel renewable alternatives in which atmospheric CO_2 is fixed directly into desired chemical compounds by the use of photosynthetic microorganisms as biotechnological hosts. Cyanobacteria have been extensively studied in this respect, and engineered for the direct autotrophic production of various potential biofuels (Oliver et al., 2016; Lau et al., 2015; Savakis and Hellingwerf, 2015) including alcohols, hydrocarbons and molecular hydrogen. Despite the scope of alternatives, bioethanol remains amongst the most promising target products due to relatively high achieved production efficiencies, and its existing status as a transport fuel in the current infrastructure.

During the past two decades various different cyanobacterial systems have been engineered and characterized for photoautotrophic ethanol production, accompanied by associated patents filed by several US-based biofuel companies such as Algenol Biofuels and Joule Unlimited [see review (Dexter et al., 2015)]. Since the first report demonstrating ethanol production in cyanobacteria (Deng and Coleman, 1999), most approaches rely on the expression of pyruvate decarboxylase (pcd) derived from the obligate ethanol producer *Zymomonas mobilis* in combination with cyanobacterial alcohol dehydrogenase (adh; encoded by *slr1192*) in either *Synechococcus* sp. PCC 7002 (*Synechococcus* 7002 from here on) or *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803 from here on). A range of engineering strategies and expression systems have been explored for improving the efficiencies (Dexter et al., 2015), with the highest obtained ethanol yields of 5.5 g L^{-1} (Gao et al., 2012) and 7.1 g L^{-1} (Dehring et al., 2012) exceeding the levels reported for any other biofuel produced in cyanobacteria. In comparison, the foremost ethanol producer *Saccharomyces cerevisiae* used in biotechnological applications has been reported to reach concentrations up to 73.8 g L^{-1} (Zhang et al., 2011), emphasizing the necessity for further development towards economically competitive autotrophic cyanobacterial production platforms.

End-product toxicity is one of the key considerations when

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designing biotechnological platforms for the continuous production of chemicals, as accumulation of the products can induce various adverse effects to the host cell even at relative low concentrations. While there appear to be clear strain-specific differences in the tolerance of cyanobacteria towards different chemicals, ethanol has been shown to be less toxic to *Synechocystis* 6803 and *Synechococcus elongatus* PCC 7942 (*Synechococcus* 7942 from here on) than longer chain alcohols such as butanol, heptanol or dodecanol (Kämäräinen et al., 2012). It has been reported that for *Synechocystis* 6803, supplementation of ethanol at 12 g L^{-1} concentration results in 50% growth reduction, accompanied by a common stress response with extensive changes in the overall protein expression profile already at short-term exposure (Qiao et al., 2012). Observed changes included the upregulation of heat-shock proteins and proteins associated with modulating the lipid composition of cellular membranes, induction of various transporters, other transmembrane proteins, and cell mobility-related proteins, in addition to the enhanced expression of numerous components involved with photosynthesis (Qiao et al., 2012). In contrast, prolonged exposure to lower concentrations of ethanol produced by the cells, as monitored at the transcript level in response to 18-day accumulation of ethanol up to 0.27 g L^{-1}, did not induce any comprehensive stress response in *Synechocystis* 6803 (Dienst et al., 2014). Although the growth of the cells was clearly retarded and bleaching could be observed, the transcript-level expression changes were confined to a few specific targets including genes coding for alcohol dehydrogenase adhA (*slr1192*), photosynthetic light-harvesting pigment protein cpcB (*sll1577*) and 30S ribosomal protein S8 (*sll1809*). These two parallel scenarios clearly demonstrate the importance of maintaining a balance between the production levels and specific cellular toxicity effects, in context with the designated analytical systems in evaluating the performance of any specific system under development.

The aim of this study was to compare the tolerance of a range of selected cyanobacterial strains and substrains towards increasing concentrations of ethanol supplemented in the medium, in order to determine strain-specific differences in regards to toxicity limits and associated growth effects. The study was expected to expand the understanding on the potential and limitations associated with the choice of most prominent strains, and associated considerations in the direct quantitative comparison of the ethanol tolerance under varying culture conditions.

2. Materials and methods

2.1. Cyanobacterial strains

The cyanobacterial strains used in the study were *Synechococcus* 7942, *Synechococcus* 7002 and five different substrains of *Synechocystis* 6803. In addition, ethanol-producing *Synechocystis* 6803 variant SAA012, and SigE over-expression strain were included in the comparison. All the strains have been listed in Table 1 with specific descriptions and references.

2.2. Culture media and cultivation conditions

All reagents were purchased form Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. *Synechocystis* 6803 and *Synechococcus* 7942 were grown in BG11 medium (Rippka et al., 1979) buffered with TES-KOH to pH 8.0, with 50 mg L^{-1} kanamycin added in the SAA012 and SigE over-expression strain cultures. By default, these strains were cultivated at 30 °C under continuous white light illumination 60 µmol photons $m^{-2} s^{-1}$ (culture condition II). *Synechococcus* 7002 was grown in A + medium (Stevens et al., 1973) supplemented with 4 µg L^{-1} vitamin B12, by default at 37 °C under continuous illumination 100 µmol photons $m^{-2} s^{-1}$ (culture condition I). The liquid cultures were incubated in a cultivation cabinet (Algaetron AG230-ECO) with 1% CO_2 atmosphere in 150 rpm shaking. Solid cultures were grown on corresponding plates containing 1.5% agar (Bioline USA Inc., Taunton, MA, USA, or Becton, Dickinson and Company, Sparks, MD, USA) and 0.3% sodium thiosulfate in MLR-351 (SANYO Electric Co., Osaka, Japan) culture chamber.

2.3. Batch cell cultivations

Pre-cultures (20 ml) inoculated from freshly prepared plates were cultivated in 100 ml Erlenmeyer flasks, adjusted to optical density at 750 nm (OD_{750}) ~1, and re-diluted the next day to OD_{750} 0.3 at the beginning of the main cultures. In the ethanol tolerance trials, 99.5% ethanol (ETAX Aa, Altia Oy, Rajamäki, Finland) was added in the cultures to the final concentrations in the selected range (0–27.6 g L^{-1}). The cultures (20 ml) were further supplemented with 50 mM of sodium bicarbonate, and sealed in air-tight 160 ml culture bottles capped with butyl rubber stoppers to avoid ethanol evaporation during the incubation. The batch cultivations in the absence of ethanol were carried out in 20 ml volume in 100 ml Erlenmeyer flasks.

2.4. Continuous photobioreactor cultures

Continuous cultivations were performed in 400 ml FMT 150 flat-panel photobioreactors (Photon Systems Instruments, Brno, Czech Republic). The system was operated in a chemostat mode with the dilution rate set to 0.18 day^{-1}, and the growth of the cells was continuously monitored by the integrated densitometer at OD_{735}. The cultures were bubbled with air and incubated in each case at the strain-specific optimum temperature (37 °C for *Synechococcus* 7942 and 30 °C *Synechocystis* 6803) under 200 µmol photons $m^{-2} s^{-1}$ continuous white light. The ethanol concentration in the cultures was increased in a step-

Table 1
Cyanobacterial strains and substrains used in this study. The table shows the associated names and abbreviations, the initial source of the material in the Molecular Plant Biology (University of Turku, Finland) library, and the primary literature references.

Strains/ substrains	Initial origin of strain or plasmid	References
Synechocystis sp. PCC 6803 substrain A [control for SAA012]	Aaron Kaplan, University of Jerusalem, Israel	See Zavřel et al. (2017)
Synechocystis sp. PCC 6803 substrain B (GT-V; Vermaas) [control for SigE OE]	Wolfgang Hess, University of Freiburg, Germany	Trautmann et al. (2012)
Synechocystis sp. PCC 6803 substrain C (PCC-M; Moscow)	Sergey Shestakov, Moscow State University, Russia	Trautmann et al. (2012), See Zavřel et al. (2017)
Synechocystis sp. PCC 6803 substrain D	Norio Murata, National Institute for Basic Biology, Japan	Kanervo et al. (1995)
Synechocystis sp. PCC 6803 substrain E	Tony Pembroke, University of Limeric, Ireland	
Synechococcus sp. PCC 7002	John Golbeck, Penn State Science, USA	
Synechococcus elongatus PCC 7942	Susan Golden, UC San Diego, USA	
Synechocystis sp. PCC 6803 SigE OE strain [cf. substrain B]	Masahiko Ikeuchi, University of Tokyo, Japan (SigE over-expression plasmid)	Osanai et al. (2011)
Synechocystis sp. PCC 6803 SAA012 strain, [cf. substrain A]	Klaas Hellingwerf, University of Stanford, USA	Savakis et al. (2013)

wise manner from zero to 55.3 g L^{-1} at 9.2 g L^{-1} intervals. Prior to each step, the growth of the cells was allowed to equilibriate, and the ethanol concentration was not increased until the value had been stable at least for 24 h. Axenity of the cultures was monitored on LB agar plates inoculated at regular intervals throughout the photobioreactor cultivation.

2.5. Determining the GI_{50} values

Growth inhibition parameter GI_{50} used for the comparison of ethanol toxicity was defined as the ethanol concentration at which the cell growth was reduced to half over a 24 h batch cultivation period. The GI_{50} values were approximated based on the recorded cell growth (OD_{750}) plotted against increasing concentrations (0–27.6 g L^{-1}) of supplemented ethanol as shown in Supplementary Fig. S1.

2.6. Spectrophotometric analysis

Growth of the cells in the batch cultures was monitored spectrophotometrically using Genesys 10S UV–vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). Absorption spectra (375–750 nm; normalized to 550 nm) were recorded using Olis CLARiTY 17 UV/VIS/NIR spectrophotometer (OnLine Instrument Systems, Inc.) from 48 h main cultures which were adjusted to OD_{750} 0.25 prior measurements.

2.7. Ethanol quantitation

For evaluating possible ethanol consumption by the cyanobacterial strains over 120 h incubation, the ethanol concentration in the growth medium was measured from three parallel replicates before and after the culture using a commercial K-ETOH Ethanol Assay Kit (Megazyme International, Bray, Ireland) according to the manufacturer's instructions.

2.8. Dry cell weight determination

Dry weight of the cyanobacterial cells (DCW) was determined from 48 h open-flask batch cultivations in triplicates, and calculated as g L^{-1} per the specific OD_{750}. The cells were filtered through a pre-weighted 1 × 0.45 µm, 25 mm diameter Durapore® PVDF filters (Millipore, Billerica, MA, USA), dried at 110 °C for 24 h in an oven, and weighted using an analytical scale (Mettler ToledoXA105 DualRange).

2.9. Transmission electron microscopy

Transmission electron microscopy (TEM) imaging for the cyanobacterial cells was performed using JEM-1400Plus Transmission Electron Microscope (JEOL USA Inc.) at 80 kV. Pelleted cells (1–10 ml culture samples) were fixed with glutaraldehyde s-collidine buffer (5%), postfixed with osmium tetroxide (OsO_4) (2%) containing potassium (3%) ferrocyanide, dehydrated with ethanol, and flat embedded in a 45359 Fluka Epoxy Embedding Medium kit. Prepared sections (ultramicrotome, 70 nm) were stained using uranyl acetate and lead citrate. Images (1.5 kx, 20 kx and 50 kx magnification) were analyzed with Fiji image processing software, Fiji open-source platform for biological image analysis (Schneider et al., 2012).

2.10. Estimation of relative cell sizes

Relative sizes of the cyanobacterial cells were estimated based on the TEM images (1.5 kx magnification), by measuring the cross sectional area of approximately 200 cells per strain with the Fiji image processing software, and averaging the maximum values representing the mid-cross sections (n = 50).

3. Results and discussion

3.1. Cyanobacterial strains selected for the comparison of ethanol tolerance

Altogether nine cyanobacterial strains and substrains were selected as the targets for the ethanol tolerance study (Table 1). The primary candidates were three established model species and representatives of most extensively studied unicellular cyanobacterial genera: *Synechocystis* 6803 and *Synechococcus* 7942 widely distributed in freshwater environments, and the marine strain *Synechococcus* 7002. Altogether, five distinct glucose-tolerant (Williams, 1988) *Synechocystis* 6803 substrains originating from different research laboratories (Table 1; substrains A—E) were included in the comparison in order to evaluate possible functional differences between commonly used parallel strains. Additionally, two engineered *Synechocystis* 6803 mutant strains which were of specific interest in context with ethanol production were introduced as additional targets: Ethanol-producing strain SAA012 carrying the expression cassette for pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhII) from *Zymomonas mobilis* (Savakis et al., 2013), and the group 2 σ factor SigE over-expression mutant, which appears to exhibit potentially enhanced metabolic flux towards pyruvate, the primary precursor for ethanol biosynthesis (Osanai et al., 2011).

3.2. Cyanobacterial strains exhibit significant differences in tolerance towards ethanol

In the primary comparison, the selected nine cyanobacterial strains (Table 1) were subjected to different concentrations of ethanol in the range 0–27.6 g L^{-1} (corresponding to 3.5% V/V or 573 mM), and analyzed for growth as the increase of the OD_{750} over a 24 h batch cultivation (Supplementary Fig. S1A–I). From this data the ethanol tolerance of each of the strains was estimated and presented as a measure of growth inhibition parameter, GI_{50}, which represents the ethanol concentration at which the growth is reduced to half in comparison to cultures without supplemented ethanol (Fig. 1). Comparison of the cyanobacterial strains in respect to the obtained GI_{50} values showed relatively broad variation in the strain-specific ethanol tolerance (Fig. 1), which in the case of the substrains supported the general conception that cyanobacteria may diverge relatively rapidly under laboratory conditions (Morris et al., 2017; Morris et al., 2014; Kanesaki et al., 2012; Trautmann et al., 2012). Clearly the least tolerant of the nine tested strains was *Synechococcus* 7942 (3 g L^{-1}), in line with previously reported relative sensitivity to chemical additives including

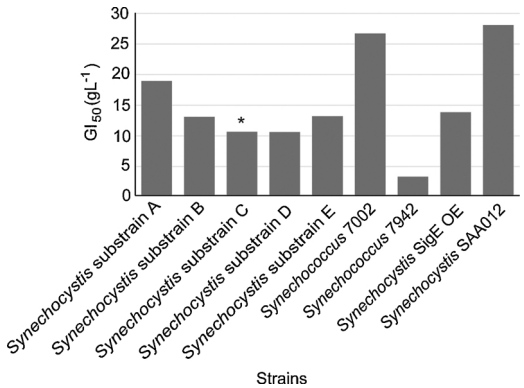

Fig. 1. Ethanol tolerance of the cyanobacterial strains and substrains compared in the study. The bars represent the GI_{50} values, which correspond to the ethanol concentration at which the growth over a 24 h cultivation period is reduced to half in comparison to control cultures without supplemented ethanol. The GI_{50} values have been obtained from on the averaged data presented in Supplementary Fig. S1. The star * indicates that reliable OD-based comparison is compromised by cell aggregation in liquid culture.

ethanol, hexane, undecane and laurate (Kämäräinen et al., 2012). The *Synechocystis* 6803 substrains B,C,D and E were all in the GI_{50} range 11–13 g L^{-1}, although in the case of the motile substrain C (Moscow or PCC-M) the OD-based analysis was somewhat compromised by cell aggregation during the liquid cultivation (see Supplementary Figs. S1C and S2). This phenotypic clumping effect has been suggested to be linked with the inactivation of the haemolysin-like surface protein HlyA (*sll1951*) potentially influencing the adsorption of toxic compounds (Sakiyama et al., 2011), and the truncation of yet uncharacterized surface protein (*slr1753*), as identified in genome sequence comparison (Trautmann et al., 2012). The most tolerant wild-type strains were *Synechococcus* 7002 and *Synechocystis* 6803 substrain A with GI_{50} values 28 and 19 g L^{-1}, respectively. Altogether, these values are in the same magnitude as reported for typical *Saccharomyces cerevisiae* strains (~55–70 g L^{-1}) (Aguilera et al., 2006), implicating that the current cyanobacterial systems are likely to be limited also by other constraints than merely the ethanol tolerance *per se*. The group 2 σ factor SigE mutant, which was included in the comparison due to reported alterations in central carbon metabolism that could potentially favor ethanol production via pyruvate (Osanai et al., 2011), appeared not to differ significantly in ethanol sensitivity from the WT background strain (substrain B). This suggests that the expression-level changes resulting from SigE over-expression do not inflict in any clear disadvantage for the cell in this respect, and that the strain could provide a prominent background for future engineering work. The ethanol-producing *Synechocystis* 6803 mutant SAA012 carrying the expression cassette for pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhII) from *Zymomonas mobilis* integrated at the genomic locus *slr0168* under the constitutive promoter *P*t5 (Savakis et al., 2013), however, appeared to be substantially more tolerant than the closest wild-type strain (substrain A) and in the same GI_{50} range with *Syncehococcus* 7002. This implies that the introduced genetic modifications in SAA012, and the resulting constant long-term exposure to intracellular ethanol, may have exerted selective pressure towards adaptation to the presence of ethanol, thus improving the potential capacity of the strain as a production host. The specific molecular basis for the increased tolerance is currently unclear, but knowing the native tendency of cyanobacteria to readily evolve in response to different types of stimuli, may be a combination of changes in expression patterns (Qiao et al., 2012; Dienst et al., 2014) via regulatory acclimation as well as genetic adaptation (microevolution).

3.3. Supplemented ethanol is not used for growth by the analyzed cyanobacterial strains

Various microorganisms are capable of utilizing available ethanol as a carbon source to sustain aerobic growth. Based on available sequence information, also cyanobacteria may be able to uptake ethanol to a certain extent, although most species lack the glyoxylate shunt (Zhang and Bryant, 2015) essential for the incorporation of acetyl-CoA –derived metabolites into the central carbon metabolism via gluconeogenesis. *Synechocystis* 6803, for example, harbors the genes required for the conversion of ethanol into acetaldehyde (alcohol dehydrogenase *slr1192*; aldehyde reductase *slr0942*) and further to acetyl-CoA (aldehyde dehydrogenase *slr0091*), and could therefore be able to use supplied ethanol as a precursor for various biosynthetic reactions potentially promoting growth. To exclude the possibility that the outcome of the inhibition experiments would be distorted by the consumption of the ethanol by the cells, the amount of ethanol was monitored over the 120 h closed batch cultures for the two most tolerant strains *Synechococcus* 7002 and *Synechocystis* 6803 substrain A (Fig. 2). The results showed that the different concentrations of supplied ethanol remained constant throughout the incubation, confirming that the cells did not utilize available ethanol to an extent which would affect the quantitative interpretation of the data.

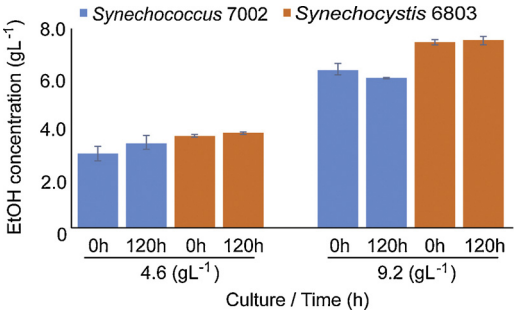

Fig. 2. Evaluation of ethanol consumption by *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 substrain A. The concentration of supplied ethanol (4.6 and 9.2 g L^{-1}) was measured for both strains at the beginning (0 h) and at the end of 120 h batch culture from three parallel replicates (n = 3).

3.4. Direct comparison of different cyanobacteria is complicated by strain and condition-specific features

Based on the initial toxicity assay, the two most ethanol-tolerant cyanobacterial strains, *Synechococcus* 7002 and *Synchocystis* 6803 (substrain A) were selected for more detailed evaluation. The strains were first analyzed in respect to their optical properties in order to validate the use of spectrophotometric measurements as grounds for quantitative comparison. The cultures were incubated for 48 h under the optimal conditions set for each strain (37 °C and 100 µmol photons m^{-2} s^{-1} versus 30 °C and 60 µmol photons m^{-2} s^{-1}), and compared for OD_{750} and dry cell weight (Table 2). The results showed that the total cell mass per absorbance was significantly lower for *Synechocystis* 6803 than for *Synechococcus* 7002 (Table 2), which implies that the relative tolerance of *Synechococcus* 7002 represented in respect to cell mass would be markedly higher than anticipated based on the OD_{750} comparison (c.f. data in Fig. 1). The culture conditions also had a clear impact on the DCW : OD_{750} ratio for each strain, with a more pronounced effect for *Synechococcus* 7002 for which the ratio increased by over two-fold upon change to lower temperature and lower light (Table 2). To elucidate these differences further, the strains grown under the same set-up were subjected to transmission electron microscopy (TEM) for cell-size comparison (Table 3) and absorbance spectrum analysis (Fig. 3). Besides the obvious and expected morphological features of the two strains, TEM showed that the overall size of the cells was always larger when grown under the strain-specific default condition, with the average cell-size of *Synechococcus* 7002 being systematically smaller in comparison to *Synechocystis* 6803 (cross sectional area ~60–90%) (Table 3; Supplementary Figs. S3–S4). Spectrophotometric analysis conducted for the same samples showed that the

Table 2
Comparison of *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 substrain A in respect to optical density (OD_{750}), dry cell weight (DCW), and the ratio between DCW and OD_{750} (OD_{750} per mgL^{-1}). The cells were cultured either at 37 °C under 100 µmol photons m^{-2} s^{-1} light (condition I) or at 30 °C under 60 µmol photons m^{-2} s^{-1} light (condition II) in three parallel biological replicates (n = 3).

Growth condition	Strain	DCW (gL^{-1})	OD (OD_{750})	DCW per OD (gL^{-1} per OD_{750})
Condition I	*Synechococcus* 7002	2.3 ± 0.28	2.1 ± 0.25	1.10
Condition I	*Synechocystis* 6803	1.4 ± 0.33	2.3 ± 0.40	0.61
Condition II	*Synechococcus* 7002	1.8 ± 0.15	0.8 ± 0.05	2.25
Condition II	*Synechocystis* 6803	0.8 ± 0.05	1 ± 0.1	0.8

Table 3
Comparison of cell sizes between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 substrain A cultured under different conditions. The cells were cultured at 37 °C under 100 µmol photons $m^{-2} s^{-1}$ light (condition I) and at 30 °C under 60 µmol photons $m^{-2} s^{-1}$ light (condition II) followed by transmission electron microscopy imaging and subsequent analysis with Fiji image processing software to obtain average estimates of relative cell sizes (n = 50) presented in arbitrate units (au). The star * indicates the default condition for each strain.

Growth condition (Relative % to*)	*Synechococcus* 7002 Cell cross sectional area (au)	*Synechocystis* 6803 Cell cross sectional area (au)
Condition I	2.24 ± 0.11 *	2.52 ± 0.04
Condition II	2.00 ± 0.08	3.01 ± 0.06 *
(Relative % to*)	89 %	83 %

reduction in temperature and light intensity also had a clear impact on the pigment profiles between the strains, with an increase in the overall pigment content (per OD_{750}) in *Synechococcus* 7002 (Fig. 3A), and an increase in the phycobilisome : chlorophyll ratio in *Synechocystis* 6803 (Fig. 3B). A subsequent analysis carried out to evaluate the effect of ethanol concentration on cell morphology revealed that the size of *Synechococcus* 7002 cells decreased in proportion to the amount of supplemented ethanol, while such direct correlation was not observed for *Synechocystis* 6803 (Table 4). Altogether, these results collectively emphasize the complications associated with the reliable quantitative comparison between different cyanobacterial strains and cultivation set-ups under varying conditions based on OD_{750}, chlorophyll content or DCW, as the selected normalization approach may have a significant effect on the interpretation of the results.

3.5. Contionuous photobioreactor cultures demonstrate relatively high tolerance for supplemented ethanol

In order to further assess the potential biotechnological capacity and limitations of the two most ethanol tolerant cyanobacterial strains, the wild-type *Synechococcus* 7002 and *Synechocystis* 6803 (substrain A) were subjected to a three-week continuous photobioreactor cultivation with a step-wise increase in the concentration of supplemented ethanol. The experiment was carried out in chemostat mode in 400 ml FMT 100 flat-panel photobioreactor under the optimal growth temperature specific for each strain, using four different ethanol concentrations between 0–36.9 g L^{-1}. After reaching a 24 h steady-state under each condition, the final culture OD was recorded as the output (Fig. 4), followed by increase in the ethanol concentration in the medium. The obtained values represented the cell densities at which the specific growth rate matched the preset dilution rate (0.18 day^{-1}), and although not directly equivalent to the GI_{50} parameter used for initial comparison, they served as an alternative measure for ethanol toxicity.

Table 4
Comparison of cell sizes between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 substrain A cultured under increasing ethanol concentrations. The cells were cultured in 48 h batch culture in sealed bottles in the presence of 0–36.8 g L^{-1} ethanol, subjected to TEM imaging and subsequent analysis with Fiji image processing software to obtain average estimates of relative cell sizes (n = 50) presented in arbitrate units (au).

Ethanol concentration in culture (gL^{-1})	*Synechococcus* 7002 Cell cross sectional area (au) (% relative to 0 g L^{-1} reference)	*Synechocystis* 6803 Cell cross sectional area (au) (% relative to 0 g L^{-1} reference)
0	2.69 ± 0.09 (100 %)	2.92 ± 0.09 (100 %)
9.2	2.42 ± 0.10 (90 %)	3.39 ± 0.12 (116 %)
18.4	2.17 ± 0.08 (81 %)	3.36 ± 0.12 (115 %)
36.8	2.06 ± 0.08 (77 %)	2.87 ± 0.12 (98 %)

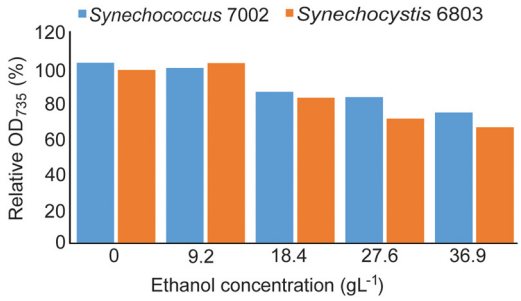

Fig. 4. Comparison of ethanol tolerance between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 in a continuous three-week photobioreactor cultivation under increasing ethanol concentrations (0–36.9 g L^{-1}). The system (FMT 150) was operated in a chemostat mode with dilution rate set to 0.18 day^{-1}, and the bars represent the final optical densities (%; normalized to maximum) after reaching a 24 h steady-state at each ethanol concentration.

The results were in line with the batch culture findings (Supplementary Figs. S1 and 1), and demonstrated that the *Synechococcus* 7002 was less affected by the presence of ethanol in comparison to *Synechocystis* 6803. Altogether, 9.2 g L^{-1} of supplemented ethanol appeared not have any impact on the monitored growth parameters for either of the strains, while further increase in the ethanol amount in the medium steadily reduced the biomass accumulation to about 75% and 65% at 36.9 g L^{-1} for *Synechococcus* 7002 and *Synechocystis* 6803, respectively. Taking into account the different optical properties and relative growth rates of the two strains (Table 2), the results suggested that the ethanol

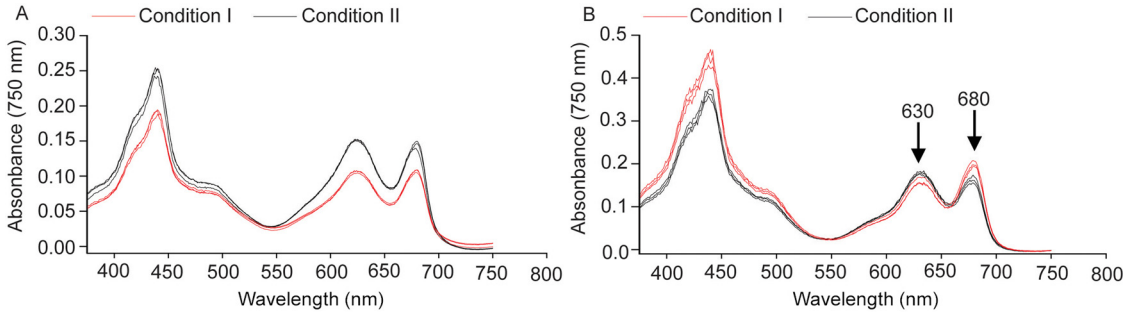

Fig. 3. The absorbance spectra (350–750 nm) a) *Synechococcus* sp. PCC 7002 and b) *Synechocystis* sp. PCC 6803 substrain A. The cells were cultured for 48 h at 37 °C under 100 µmol photons $m^{-2} s^{-1}$ light (condition I; red line) or at 30 °C under 60 µmol photons $m^{-2} s^{-1}$ light (condition II; black line) and measured in three parallel biological replicates (n = 3). The absorption maxima for phycobilisomes (630 nm) and chlorophyll a (680 nm) have been indicated by arrows (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

tolerance of *Synechocystis* 6803 – approximated in respect to relative cell mass – is likely to be less than half of that of *Synechococcus* 7002.

4. Concluding remarks

While cyanobacteria appear not to be particularly sensitive to ethanol, the results show that there are rather extensive differences in ethanol tolerance between different strains and substrains that originate from different research laboratories. Out of the strains included in this study, *Synechococcus* 7002 and *Synechocystis* 6803 (substrain A) were most tolerant, and showed no obvious response towards ethanol at concentrations below 9.2 g L^{-1} (1.2% V/V), with GI_{50} values 28 g L^{-1} (3.5% V/V) and 19 g L^{-1} (2.4% V/V), respectively. The highest reported ethanol production yields in cyanobacteria are currently in the range 7 g L^{-1} (~0.9% V/V), and thus below the observed threshold, yet the ongoing development in the field and enhancements in productivity will eventually require strains which are less affected by ethanol. The results also emphasize strain-specific and condition-specific variation in optical properties, pigment content and cell morphology of cyanobacteria, which makes direct quantitative comparison of the parallel cultures challenging.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.07.034.

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