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Impact of *glgA1*, *glgA2* or *glgC* overexpression on growth and glycogen production in *Synechocystis* sp. PCC 6803

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ABSTRACT

Low production rates are still one limiting factor for the industrial climate-neutral production of biovaluable compounds in cyanobacteria. Next to optimized cultivation conditions, new production strategies are required. Hence, the use of established molecular tools could lead to increased product yields in the cyanobacterial model organism *Synechocystis* sp. PCC6803. Its main storage compound glycogen was chosen to be increased by the use of these tools. In this study, the three genes *glgC*, *glgA1* and *glgA2*, which are part of the glycogen synthesis pathway, were combined with the P_{cpc560} promoter and the neutral cloning site NSC1. The complete genome integration, protein formation, biomass production and glycogen accumulation were determined to select the most productive transformants. The overexpression of *glgA2* did not increase the biomass or glycogen production in short-term trials compared to the other two genes but caused transformants death in long-term trials. The transformants *glgA1*_11 and *glgC*_2 showed significantly increased biomass (1.6-fold - 1.7-fold) and glycogen production (3.5-fold - 4-fold) compared to the wild type after 96 h making them a promising energy source for further applications. Those could include for example a two-stage production process, with first energy production (glycogen) and second increased product formation (e.g. ethanol).

1. Introduction

Cyanobacteria represent a promising system as microbial cell factories for climate-neutral production of valuable compounds. These organisms fix atmospheric CO_2 using solar energy and nutrients from the environment and convert it into various useful products (Lakatos et al., 2019). The low productivity compared to other existing manufacturing systems, is still a limiting factor and thus, the development of new production strategies is needed (Lopes da Silva et al., 2018; Noreña-Caro and Benton, 2018). These include optimization of media components, design of photobioreactor system, process parameters and genetic strain optimization (Kamravamanesh et al., 2018). Years of research have led to many genetic tools that are used for genomic modifications of these microorganisms, to increase the production of naturally occurring metabolites like glycogen or to integrate new metabolic pathways to produce foreign substances like bioethanol (Du et al., 2018). One of these well characterized microorganisms is the cyanobacterial strain *Synechocystis* sp. PCC6803 (further as *Synechocystis*), which has been used in numerous studies on metabolic engineering and synthetic biology (Arisaka et al., 2019; Armshaw et al., 2015; Ferreira et al., 2018; Gundolf et al., 2019; Liu and Pakrasi, 2018; Ng et al., 2015; Tony Pembroke et al., 2019; Wang et al., 2018). As a result, its genome has been completely sequenced (Kaneko et al., 1996) and several molecular tools like various promoters and neutral cloning sites (NSC) have been established, as summarized by Sun et al. (2018) and Gundolf et al. (2019). Fifteen NSCs in the genome of *Synechocystis* were identified; two of them have been well characterized. NSC1 was the most suitable one for long-term stable genomic integration (Ng et al., 2015). From twelve different promoters tested, the endogenous, light-inducible P_{cpc560} promoter showed the highest activity (Liu and Pakrasi, 2018), which was

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confirmed by other studies as well (Behle et al., 2019; Ng et al., 2015; Zhou et al., 2014).

Here, the glycogen pathway was closely investigated as glycogen is the main central storage compound in *Synechocystis.* Therefore, it could be used as an energy source and a precursor supply as its overproduction could be beneficial for the increased production of a desired compound within a two-stage production process within one strain. In the first stage, glycogen could be overproduced to obtain more biomass and precursors, the later could be converted into a more complex product in a second production stage (Gundolf et al., 2019). The different synthesis steps could be controlled using different types of inducible promoters. Since P_{cpc560} is light-inducible, gene expression is highest at the beginning of the growth phase, while its activity decreases with increasing cell density (Ng et al., 2015). This promoter could be used to produce glycogen in the first stage (growth phase) of the process. The second stage (production stage of the desired compound) could be activated by an additive like metal ions or isopropyl-β-D-thiogalactopyranosid (IPTG) (Gundolf et al., 2019). Similar two-stage processes have already been shown in bacteria and yeast using inducible promoters to overcome the negative growth effects of toxic compounds like ethanol related with reduced production yields (Dühring et al., 2006; Lee et al., 2010; Małachowska and Olszewski, 2018; Nevalainen et al., 2005). As the product yield is dependent on the initial cell density and precursor availability, the aim of this study was the generation of *Synechocystis*-transformants, which would allow for clearly increased biomass and glycogen production for the first part of such a two-stage production process.

In order to increase the energy and precursor supply in *Synechocystis,* the accumulation of the central storage compound glycogen could be enhanced by the overexpression of the three main genes involved in the glycogen synthesis pathway. These are the gene for ADP-glucose pyrophosphorylase (*glgC*), catalyzing the formation of the ADP-glucose from glucose-6-phosphate, and the two genes for the glycogen synthases (*glgA1* and *glgA2),* catalyzing the elongation of a glucose chain resulting in the primary structure of glycogen (Díaz-Troya et al., 2014). The protein encoded by *glgC* catalyzes the first step of glycogen synthesis and was published to have a major influence on glycogen synthesis since its gene deletion causes the absence of glycogen in *Synechocystis* (Miao et al., 2003). Besides *glgC,* the simultaneous knockout (KO) of *glgA1* and *glgA2* also led to the complete loss of glycogen production in this organism (Gründel et al., 2012).

So far, genomic modifications and changes in cultivation parameters have been performed to increase glycogen production in several cyanobacterial strains like *Synechococcus* sp. and *Synechocystis* sp. (Comer et al., 2020; Gupta et al., 2020; Hickman et al., 2013; Qiao et al., 2018; Velmurugan and Incharoensakdi, 2018). Within the following section, we focus on *Synechocystis* sp. PCC6803 to ensure a valid comparison of the achieved results regarding the glycogen amounts. Especially, the cultivation parameters play an important role as glycogen production can be induced in nutrient limitation due to nitrogen or phosphate deficiency (Lakatos et al., 2019; Yoo et al., 2007). Velmurugan and Incharoensakdi (2018) showed that the combined overexpression of *glgA1* and *glgC* controlled by the P_{psbA2} promoter resulted in 34.8% glycogen (g g^{-1} dry cell weight; DCW) instead of 24.1% in the wild type (WT) after 20 d of cultivation. In addition, the KO of the competing metabolic pathway polyhydroxybutyrate (PHB) increased the glycogen content to 38.3%. Under stress conditions, an increase from 27% after 13 d in standard medium to 36% after 10 d in standard medium with an additional three days in medium without nitrogen could be observed (Velmurugan and Incharoensakdi, 2018). Here, the influence of the genes *glgA1*, *glgA2* and *glgC* in combination with the P_{cpc560} promotor and the NSC1 is investigated and compared among each other and the WT in regard to the biomass and glycogen production. This combination of genetic tools has not been applied to this metabolic cascade before and thus new insights into the function of these genes, as well as information about the applicability of the used genetic tools within *Synechocystis*, is gained.

2. Materials and methods

2.1. Strains and culture cultivation

The WT strain *Synechocystis* obtained from the laboratory of Prof. Peter Nixon (Imperial College London) was cultivated at 30 °C under continuous illumination of 50 µmol photons m^{-2} s^{-1} in BG11 medium (Stanier et al., 1971) supplemented with 10 mmol L^{-1} $NaHCO_3$. To accelerate the growth for genetic optimization (transformation), the $NaHCO_3$ supply was increased to 50 mmol L^{-1} in pre-cultures. For screening on the DNA level, the engineered strains (transformants) were cultivated on BG11 agar plates (1.5%) supplemented with 20 mmol L^{-1} HEPES (for pH stabilization) before autoclaving, and 10 mmol L^{-1} $NaHCO_3$, and 5–25 µg mL^{-1} of spectinomycin (Spe) after autoclaving at 30 °C under continuous illumination at 10–20 µmol photons m^{-2} s^{-1}. The same concentration of Spe was added to transformants grown in BG11 medium. For the protein determination, the transformants were cultivated in 12-well plates using the mentioned cultivation conditions. The strains were cultivated in a CFL plant climatics cultivation chamber with cool fluorescent tubes (TL-D 18W/820, Philips, Germany) for transformation as well as for screening on the genomic and proteomic level.

For the growth characterization of the transformants compared to the WT, pre-cultures were inoculated from agar plates and then cultured in 400 mL sterile glass columns using 350 mL BG11 medium at 30 °C using a temperature-controlled water bath. A picture of the used cultivation system is shown in the Supplementary Data (Fig. S1). The cultures were gently mixed by bubbling air supplemented with 2% CO_2 from the bottom of the cultivation columns. The light intensity was increased stepwise from 30 to 100 µmol photons m^{-2} s^{-1} to avoid light stress. Photosynthetically active radiation (PAR) was provided continuously from one side through a horizontally placed panel using high-frequency cool fluorescent tubes (36 W/830 Lumilux, Osram, Germany). The irradiance intensity reaching the surface of the cultivation columns was measured by a quantum sensor (LI-190SA, cosine-corrected up to 80° angle of incidence) coupled to a light meter (LI-250, Li-Cor, USA). The pre-cultures reached the stationary growth phase (dry biomass density of approximately 1–2 g L^{-1}) in about two weeks. Then, the cultures were diluted to the initial biomass density of 0.5 g L^{-1} with fresh BG11 medium to 350 mL batches and transferred into sterile 400 mL glass columns. The cultivation conditions were kept the same. The experiment was carried out for four days in triplicates. 15 mL samples for glycogen and DCW analysis were collected every day and stored at −20 °C without refilling of the glass columns.

The *E. coli* strain XL1 blue used for the expression vector construction and isolation was obtained from the Institute of Biophysics (Linz, Austria). It was grown in LB medium (Roche) on a shaker at 170 rpm and 37 °C, or on LB agar plates (1.5%) at 37 °C in the presence of 100 µg mL^{-1} ampicillin or 50 µg mL^{-1} Spe, respectively.

2.2. Construction of the expression vectors

The endogenous genes *glgA1* (*sll0945*), *glgA2* (*sll1393*) and *glgC* (*slr1176*), also referred to as genes of interest (GOI), were amplified with the proofreading Herculase II Fusion DNA polymerase (Agilent Technologies, USA) using extracted genomic DNA from *Synechocystis* (Formighieri and Melis, 2014) according to the manufacturer protocol (Supplementary Data Tables S1 and S2). The synthesis of DNA oligonucleotides (primers) was provided by Sigma-Aldrich or Eurofins Genomics. The PCR products of the genes were extended by restriction enzyme target sites, an upstream spacer (18 bp) and one of the protein tags V5 (*glg*A2), 3xHA (*glg*A1) or S (*glg*C). The upstream (US) and downstream (DS) region of the NSC1 (Ng et al., 2015) as well as the strong cell internal P_{cpc560} promoter including a native ribosome binding site (Zhou et al., 2014) derived from genomic DNA of *Synechocystis* and a Spe resistance cassette (Spe^R) from pSYN_2 control were amplified

and extended with restriction enzymes target sites. Following restriction digest (restriction enzymes, New England Biolabs, USA) and ligation (T4 ligase, Promega, USA), the fragments were combined sequentially within four cloning steps (1. US, 2. DS+Spe^R, 3. P_{cpc560}, 4. GOI) into the pBlueskript II SK (+) (Institute of Biophysics, Austria), which was used as the plasmid backbone. Finally, three different plasmids harboring one gene, respectively, were generated (Fig. 1A). A list of these plasmids and the used primers is shown in the Supplementary Data (Tables S1 and S3). The plasmids were transformed into the *E. coli* strain XL1 blue by heat shock (Mülhardt, 2013) and isolated either by alkaline lysis (Sambrook and Russel, 2001) or by the Plasmid Miniprep Kit GeneJET (Thermo Fischer Scientific, USA). The verification of the NSC1, promoter, GOI and Spe^R integration was done by restriction digest, colony PCR using the polymerase FIREPol® DNA polymerase (Solis BioDyne, Estonia) according to the manufacturer's protocol (Tables S1 and S2) and sequencing by LGC Genomics (Germany) or Eurofins Genomics (Austria). The sequence alignment and verification were done using the software SnapGene (GSL Biotech LLC, USA, version 4.0.8).

2.3. Construction of engineered strains

The permanent integration of the generated expression vectors into the genome of *Synechocystis* was done by natural transformation according to a modified protocol of Zang et al. (2007). 50 mL *Synechocystis* culture in its exponential growth phase with OD = 0.5 at 730 nm (absolute OD = 25) was harvested at 3,000 rpm for 20 min. The cells were re-suspended in BG11 medium supplemented with 10 mmol L^{-1} $NaHCO_3$. 200 µL cell suspension was incubated with 100 ng plasmid DNA at 30 °C and 30 µmol photons m^{-2} s^{-1} for 2 h. The culture was spread on BG11 agar with 10 mmol L^{-1} $NaHCO_3$ and 20 mmol L^{-1} HEPES and incubated at the same conditions for 12–24 h to recover from the transformation. Then, the culture was transferred on a new plate containing the same medium and 5 µg mL^{-1} Spe. Single colonies were picked, transferred and grown on new agar plates to analyze the DNA and to determine the protein levels.

2.4. Determination of genomic modification

For screening of segregated transformants, the genomic integration of the fragments P_{cpc560}, GOI and Spe^R in the native NSC1 by homologous recombination was verified by PCR with the ONE Taq DNA polymerase (New England Biolabs, USA) in three consecutive steps. The amplification was either performed by colony PCR or extracted genomic DNA (Formighieri and Melis, 2014). The gene exchange of all genome copies per cell was verified by multiplex PCR using the primers 2-13F, 2-14R and 6-15F and an elongation time of 1 min 10 s. The primers 2-13F and 2-14R indicated an unmodified genome while the modified one was detected by the primers 2-14R and 6-15F. The integration of all three transgenes was examined by using the primers 2-13F and 6-16R resulting in a gene specific fragment size (elongation time: 2 min). The degradation of the expression vector within the cell was investigated with the plasmid backbone specific primer 9-11F and the primer 6-15F (elongation time: 1 min 25 s). The PCR products were separated on a 1% agarose gel in 1x TAE buffer stained with the Roti®-Gel Stain (Carl Roth, Germany). The visualization was done on the FlourChem FC3 Imager (ProteinSimple, USA) using the software AlphaView (ProteinSimple, USA, version 3.4.0.0). The size standards peq Gold 1 kb DNA Ladder (VWR Life Science, USA), FastRuler Middle Range DNA Ladder (Thermo Fischer Scientific, USA) or FastRuler High Range DNA Ladder (Thermo Fischer Scientific, USA) were used. The primer sequences and amplification parameters are summarized in the Supplementary Data (Tables S1 and S2) and their binding sites are visualized in Fig. 1A.

For sequencing of the integrated fragments, the modified region was amplified with the proofreading Kapa HiFi DNA polymerase (Roche,

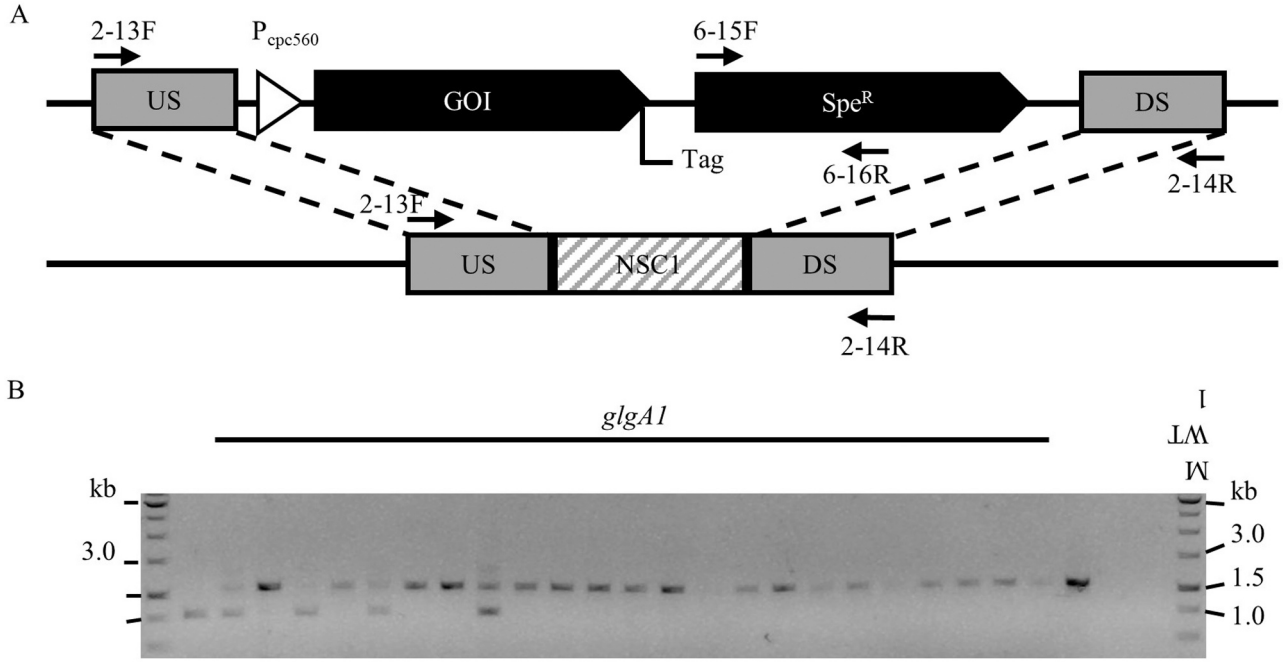

Fig. 1. Verification of complete segregation of *Synechocystis* sp. PCC6803 transformants. (A) Schematic structure of the cloned expression vector carrying the neutral cloning site (NSC1; US - NSC1 upstream, DS - NSC1 downstream) for the double homologous recombination into the genome, the P_{cpc560} promoter, the antibiotic resistance gene for spectinomycin (Spe^R), one of the genes of the glycogen synthesis pathway *glgA1*, *glgA2* or *glgC* (gene of interest; GOI) and an individual protein tag for each GOI; (B) Determination of the *glgA1* integration into all copies of the genome by polymerase chain reaction (PCR) amplification using the ONE Taq DNA Polymerase, the primers 2-13F, 2-14R and 6-15F after genomic DNA extraction followed by separation on a 1% agarose gel, showing the wild type (WT) genome specific band at 0.778 kilobasepair (kb) and the transformant genome specific band at 1.076 kb. pc - positive control (expression vector used for the transformation), nc - PCR mix without a template, M - 1 kb peqGold DNA Ladder.

Switzerland) using the primer pair 2-13F and 2-14R according to the PCR protocol summarized in the Supplementary Data (Table S2) after the extraction of genomic DNA from selected transformants (Formighieri and Melis, 2014). The amplified fragments were purified with the Wizard SV Gel and PCR Clean-Up System Kit (Promega, USA). Sequencing was performed by Eurofins Genomics and the sequence alignment and verification were carried out using the software SnapGene.

2.5. Determination of protein production

The transformant colonies were picked from the plate, cultured in 4 mL BG11 medium with 10 mmol L^{-1} $NaHCO_3$ at 30 °C and 50 µmol photons m^{-2} s^{-1} in 12-well cell culture plates for one week. 2 mL of a *Synechocystis* culture with an OD at 730 nm of 1 (absolute OD = 2) was harvested at 13,000 rpm, 4 °C for 10 min. The protein extraction and denaturation were done according to Richter (2018). The proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using a 12.5% SDS-polyacrylamide gel (Sambrook and Russell, 2006). The silver staining was performed according to Heukeshoven and Dernick (1985). The Western blot was performed by the transfer of the proteins onto a nitrocellulose membrane (Amersham™ Protran™ Premium, pore size 0.2 µm, GE Healthcare, United Kingdom) using a tank blotter (Bio-Rad, USA) and further proceeded according to the manual of the Anti-HA antibody (Roche, Switzerland). The monoclonal HRP-conjugated antibodies Anti-HA (dilution 1:1000), Anti-S (Abcam, United Kingdom, dilution 1:2000) or Anti-V5 (Sigma Aldrich, USA, dilution 1:2000) diluted in 1x Tris buffered saline (TBS) with 1% slim fat milk powder and 0,1% Tween 20 were incubated for 60 min at 37 °C followed by three washing steps with 1x TBS containing 0,1% (w/v) Tween 20 for 10 min. The visualization was done using the Clarity™ Western ECL substrate (Bio-Rad, USA) according to the manual and the image was captured on the FlourChem FC3 Imager with a chemiluminescence filter using the software AlphaView. The reference protein was a multi-tag protein from GenScript (Netherlands). The Amersham™ ECL™ Rainbow™ Marker - High range (Sigma Aldrich, USA) or the BLUeye Prestained Protein Ladder (Sigma Aldrich, USA) were used as a protein size standard.

2.6. Determination of long-term genomic stability

The stable, long-term transgene integration within the transformant genomes was confirmed by testing the stable foreign DNA integration over one month without selection pressure. The transformants were picked from agar plates and cultured for 49 days at 30 °C under continuous illumination of 20 µmol photons m^{-2} s^{-1} using the 6-well cell culture plates containing 4 mL BG11 medium supplemented with 10 mmol L^{-1} $NaHCO_3$ and either with or without 10 µg mL^{-1} Spe. The genomic DNA was extracted (Formighieri and Melis, 2014) and analyzed by PCR using the ONE Taq DNA polymerase (New England Biolabs, USA) and the primers 2-13F, 2-14R and 6-15F as well as 2-13F and 6-16R. PCR amplification as well as the visualization on a 1% agarose gel was done as described in Section 2.4.

2.7. Dry cell weight measurement

During the cultivation experiments for the growth characterizations of the transformants, the produced biomass of each cultivation unit was measured as DCW daily. Glass microfiber filters (size 55 mm, particle retention: 1.2 µm, VWR International, Sweden) were dried in an oven at 105 °C for 8 h and stored in a desiccator before their weight was measured. Pre-dried filters were mounted on vacuum filtration systems and 5 mL samples of the culture were applied. The loaded filter papers were dried at 105 °C in an oven overnight and their masses were determined. The actual dry weight of the biomass was calculated from the difference between the empty and loaded filter paper. The measurements were done in triplicate. The calculations of the space-time yield (STY) of the DCW (STY_{DCW}) per cultivation day and the average reached STY of the DCW (avg. STY_{DCW}) is shown in Eqs. (1) and (2).

$$\mathrm{STY_{DCW}}\left(\mathrm{gL^{(-1)}d^{(-1)}}\right) = \frac{\mathrm{g\ L^{-1}\ DCW}}{\mathrm{process\ time\ in\ d^{-1}}} \quad (1)$$

$$\mathrm{avg.\ \ STY_{DCW}}\left(\mathrm{g\ \ L^{-1}d^{-1}}\right) = \frac{STY_{DCW\ \ (24h)} + STY_{DCW\ \ (48h)} + STY_{DCW\ \ (72h)} + STY_{DCW\ \ (96h)}}{4} \quad (2)$$

2.8. Glycogen measurement

To measure the amount of glycogen produced by the transformants, 5 mL of the daily collected culture sample was used and stored at −20 °C until processing. For sample preparation, the 5 mL culture sample was centrifuged at 4000 rpm for 10 min 4.5 mL supernatant was discarded. The pellet was re-suspended, transferred to a 2 mL centrifugation tube and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded, the wet pellet was re-suspended in 300 µL 2 mol L^{-1} HCl and incubated at 95 °C and 500 rpm for 2 h to perform acid hydrolysis, in which glycogen is converted into glucose monomers. The sample was diluted 1:6 in deionized water and filtered through a 0.45 µm PTFE syringe filter (Fisherbrand, USA). An aliquot of 20 µL was injected into a liquid chromatography system (LC, Prep 300 pump, LabAlliance, USA) equipped with a refractive index detector (Refractometer, RI2000, Schambeck SFD, Germany) using a CARBOSep COREGEL 87 H column (Transgenomic™, USA). The temperature of the column oven was set to 37 °C. A flow rate of 0.6 mL min^{-1} and 0.005 mol L^{-1} H_2SO_4 as mobile phase were used. The glucose concentration was calculated based on a five-point glucose standard calibration curve, using the concentrations 0; 0.025; 0.25; 2.5 and 25 g glucose L^{-1}. The measurements for each cultivation unit were done in triplicates. The calculations of the glycogen per DCW (Eq. 3), the STY of glycogen ($STY_{glycogen}$) per cultivation day (Eq. 4) and the average reached STY of the glycogen (avg. $STY_{glycogen}$) (Eq. 5) are shown.

$$\%\ \ \mathrm{glycogen\ \ per\ \ dry\ \ cell\ \ weight\ \ (DCW)} = \frac{\mathrm{g\ \ L^{-1}glycogen}}{\mathrm{g\ \ L^{-1}\ \ DCW}} * 100 \quad (3)$$

$$\mathrm{STY_{glycogen}}\left(\mathrm{g\ \ L^{-1}d^{-1}}\right) = \frac{\mathrm{g\ \ L^{-1}\ \ glycogen}}{\mathrm{process\ \ time\ \ in\ \ d^{-1}}} \quad (4)$$

$$\text{avg.STY}_{\text{glycogen}}\left(\text{g}\quad \text{L}^{-1}\text{d}^{-1}\right) = \frac{STY_{\text{glycogen}(24h)} + STY_{\text{glycogen}(48h)} + STY_{\text{glycogen}(72h)} + STY_{\text{glycogen}(96h)}}{4} \quad (5)$$

2.9. Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between accumulated glycogen and produced DCW of transformants and the wild type. One way ANOVA and Holm-Sidak test was conducted for every binary combination of data. *P* values lower than 0.05 were considered to be significantly different. All the experiments were carried out in triplicate as well as data records (n = 3).

3. Results and discussion

In order to optimize the production of valuable biomaterials using *Synechocystis* as a model organism, a valuable first step would be to increase the amount of the main energy storage compound glycogen in *Synechocystis* cells. This increased glycogen could subsequently be used for a two-stage production process, first showing enhanced growth and glycogen accumulation and second an efficient product synthesis using an inducible promoter to drive gene expression (Gundolf et al., 2019). Therefore, the influence of the genes *glgA1*, *glgA2* and *glgC* in combination with the P_{cpc560} promotor and the NSC1 on the accumulation of biomass and glycogen production was investigated.

3.1. Transformant generation

Synechocystis is a model organism and thus, is well-characterized. There have already been numerous molecular tools identified, that can be used to achieve increased gene expression and/or product formation (Gundolf et al., 2019). For stable long-term production, NSC1 has proven to be the most productive integration site for gene expression (Ng et al., 2015). The P_{cpc560} promoter, derived from *Synechocystis* itself, leads to the highest protein production known to date with up to 15% of the total soluble protein (Zhou et al., 2014). In this study, the genes *glgC*, *glgA1* and *glgA2* were overexpressed, using these molecular tools. The linearized schematic structure of these generated expression vectors is shown in Fig. 1A. These vectors were inserted into the genome of *Synechocystis* by double homologous recombination at the NSC1 region (Fig. 1A). The natural transformation and integration of the vector harboring the *glgC* gene resulted in 3 times more transformants (110 out of eight transformations) compared to *glgA1* (32 out of seven transformations) and *glgA2* (35 out of nine transformations). Since the generated expression vectors differ only in the gene to be overexpressed, no differences with respect to the integration into the genome were expected. Based on the results obtained, the *glgC* gene appears to be more effectively integrated into the genome compared to the two glycogen synthases, suggesting that the integration of *glgC* may be more beneficial for the organism. *glgC* is described as the key gene in glycogen synthesis, which would support a favored genome integration (Gründel et al., 2012; Luan et al., 2019; Velmurugan and Incharoensakdi, 2018). Moreover, it also indicates that the genomic integration at the NSC1 site may be gene dependent as the organism preferred the integration of this specific gene.

3.2. Selection of complete segregated transformants

For *Synechocystis,* stable gene expression by genomic integration of heterologous genes was shown in previous studies (Ng et al., 2015; Proels, 2014; Veetil et al., 2017). Therefore, the fragments P_{cpc560}, GOI, and Spe^R in each of the up to 200 genomes per cell (depending on the growth phase within used cultivation parameters) ensures stable gene expression, as the naturally occurring NSC1 sequence is knocked out (Griese et al., 2011). Thus, a reversion to the WT genotype should be impossible, allowing the applicability of NSC1 to be verified and validated regarding to long-term stable product formation. The genomes of the transformants were investigated using three consecutive PCR amplification steps. First, the KO of the WT genome sequence followed by the GOI integration at the target site (NSC1) and the degradation of the expression vectors within *Synechocystis* were examined. An example of the first PCR analysis of transformants presumably having an overexpression of *glgA1* is shown in Fig. 1B. Examples of the determination of the other two genes are shown in Fig. S2 A–B. A transformant genome-specific primer (6-15F) and the primer 2-14R were used, resulting in a specific fragment size of 1.076 kb within the applied elongation time of the PCR. This leads to a distinction of fully segregated and partly segregated transformants as the naturally occurring sequence is 0.778 kb (amplified by the primer pair 2-13F and 2-14R). As shown in Fig. 1B, only one band is visible at 1.076 kb for most transformants, confirming complete modification of all genomes. From the up to 60 examined transformants carrying either a *glgA1*-, *glgA2*- or *glgC*-overexpression, over 87% showed complete segregation. Moreover, most transformants were fully segregated without any increase in the antibiotic selection pressure. Unlike in other reports (Vermaas, 1996; Wang et al., 2013) which showed that complete segregation was only achieved via a stepwise increase of the antibiotic selection pressure. The specific investigation of the GOI integration using the primers 2-13F and 6-16R yielding the gene specific fragments of 3.163 kb for *glgC*, 3.313 kb for *glgA1* und 3.304 kb for *glgA2* are shown in Fig. S3. The specific transgene integration of *glgA1* (69%) was lower compared to the other two genes (98%). The expression vector itself (1.406 kb) could not be detected with the primer 6-15F and the backbone-specific primer 9-11F in any of the investigated transformants (examples shown in Fig. S4), confirming that the vector is not self-replicative as the origin of replication of the vector could not be used by *Synechocystis*. Only those transformants that passed all three tests were used for further experiments. The growth of the individual transformants on agar plates showed significant differences, which led to the selection of only well-growing transformants for all subsequent experiments.

3.3. Selection of protein-producing transformants

Since the integrated genes originated from *Synechocystis,* each GOI was cloned with a specific protein tag enabling a distinct detection of the recombinant protein. A qualitative analysis was carried out for the first selection of transformants. For each test, a silver stained SDS gel was prepared to visualize the proteome of the wild type and of the transformants to check the successful protein extraction, loading and separation. No visual increase in the amount of the desired protein was detected in any stained SDS gel (Fig. S5). The selected, fully segregated transformants were analyzed by Western Blot analysis and showed the expected protein masses of 51 kDa for GlgC and 58 kDa for both GlgA1 and GlgA2 (Fig. 2). The analysis of 22 *glgA1*-transformants harboring the HA-tag and 17 *glgC*-transformants harboring the S-tag resulted in a defined band that was not present in the WT (selected transformants are shown in Fig. 2A, C). An interaction of the anti-V5-antibody and the WT protein extract at the expected size of the GlgA2-protein was observed

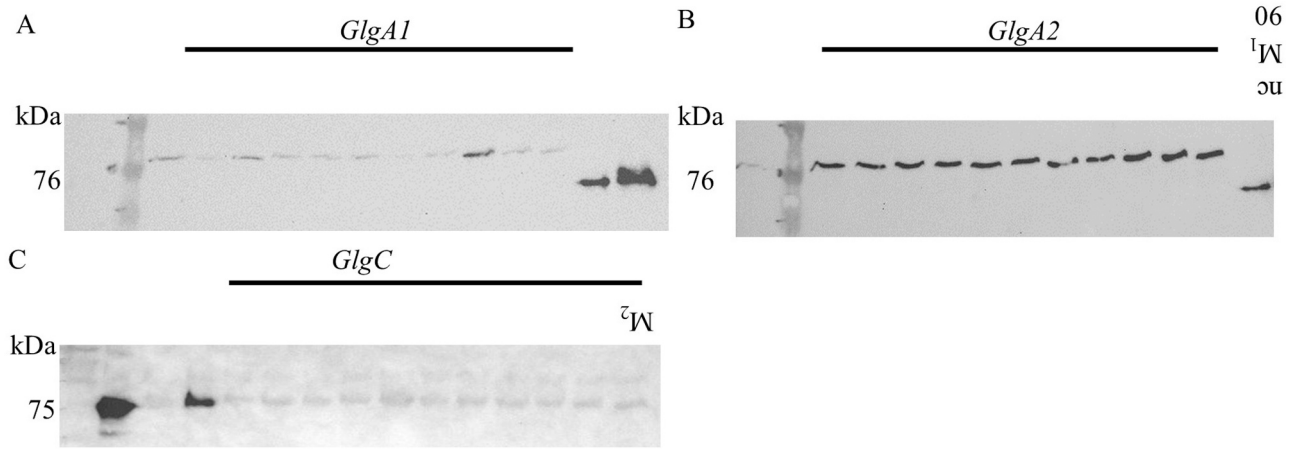

Fig. 2. Verification of the protein production of fully segregated transformants. Visualization of the produced proteins after heat extraction of the *Synechocystis* sp. PCC6803 wild type (nc; WT) or its transformants carrying an overexpression of (A) *glgA1*, (B) *glgA2*, (C) *glgC*, protein size separation with a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detection via Western Blot analysis (tank blotter) on a nitrocellulose membrane for 60 min, HRP conjugated antibody incubation for 60 min at 37 °C (A) Anti-HA-Tag (1:1000), (B) Anti-S-Tag (1:2000) or (C) Anti-V5-Tag (1:2000) and visualization using enhanced chemiluminescence and light exposure for (A, C) 10 min or (B) 20 min pc – positive control (multi-tag protein); mi – multi-tag protein's signal inhibition caused by WT matrix interaction with the antibody; kDa – kilodalton, M_1 - *Rainbow™ Marker - High range,* M_2 - *BLUeye Prestained Protein Ladder.*

(Fig. 2B). The amount of the detected protein based on signal intensity showed a more intense band in all 23 investigated *glgA2*-transformants compared to the wild type, suggesting that the integrated *glgA2* was translated into a protein. Since all integrated genes were translated into proteins, this suggests that the combination of the tools used for the different genes potentially leads to increased product formation, due to the increased gene expression. Several transformants showed promising results regarding protein synthesis from the integrated gene. Within a first selection of transformants and to show the proof of concept, the eleven transformants *glgA2*_132, *glgA2*_135, *glgA2*_143, *glgC*_2, *glgC*_6, *glgC*_159, *glgC*_160, *glgC*_162, *glgA1*_2, *glgA1*_6 and *glgA1*_11 were selected and further investigated regarding their growth and glycogen production compared to the WT. Moreover, the integrated transgene region of these eleven transformants was checked by sequencing and all of them showed complete and correct genomic integration.

3.4. Determination of long-term genomic stability

To verify *Synechocystis* as a production system for increased glycogen accumulation, stable long-term integration of the new expression cassette within all genomes per cell was required, which was previously demonstrated by Ng et al. (2015) over 30 days. In this study, complete segregation as well as transgene integration was determined for nine of the most promising transformants (*glgC*_2, *glgC*_6, *glgC*_159, *glgC*_160, *glgC*_162, *glgA1*_2, *glgA1*_6 and *glgA1*_11) after 49 days of cultivation with and without antibiotic selection pressure by PCR (Fig. 3). The transformants overexpressing *glgA2* died within the long-term cultivation trial and thus, no long-term genomic stability could be confirmed. As a control, the wild type was cultivated and tested under the same conditions. The wild type supplemented with antibiotics died under these conditions, as expected. In Fig. 3, a defined band at 1.076 kb is visible for all investigated transformants and no wild type correlated band was detected, confirming the complete segregation and loss of the natural genome sequence. Further, in Fig. S5, a distinct gene specific band (*glgA1*: 3.313 kb; *glgC*: 3.163 kb) was visible, verifying transgene integration. This suggests that all genes are permanently integrated into the host genomes, which further implies that a loss of the transgene during the production process is highly unlikely. Moreover, the stable gene integration in the absence of antibiotics offers the possibility to cultivate the transformants without selection pressure during the production process, resulting in reduced production costs.

3.5. Determination of the growth behavior

The growth of the most promising fully segregated transformants *glgA2*_132, *glgA2*_135, *glgA2*_143, *glgC*_2, *glgC*_6, *glgC*_159, *glgC*_160,

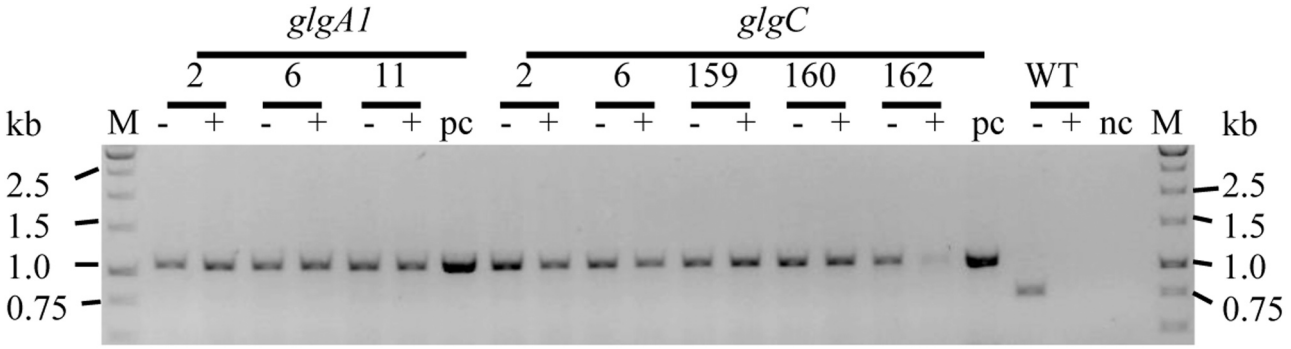

Fig. 3. Verification of the long-term genome stability of the integrated construct. Polymerase chain reaction (PCR) analysis of *glgC*- and *glgA1*-transformants after 49 days of cultivation with (+) and without (-) antibiotic in the culture medium. After genomic DNA extraction, amplification was performed using the ONE Taq DNA polymerase and the primers 2-13F, 2-14R and 6-15F. The resulting DNA fragments were separated on a 1% agarose gel, showing the wild type (WT) genome specific band at 0.778 kilobasepair (kb) and the transformant genome specific band at 1.076 kb. pc - positive control (expression vector used for the transformation), nc - PCR mix without a template, M - 1 kb peqGold DNA Ladder.

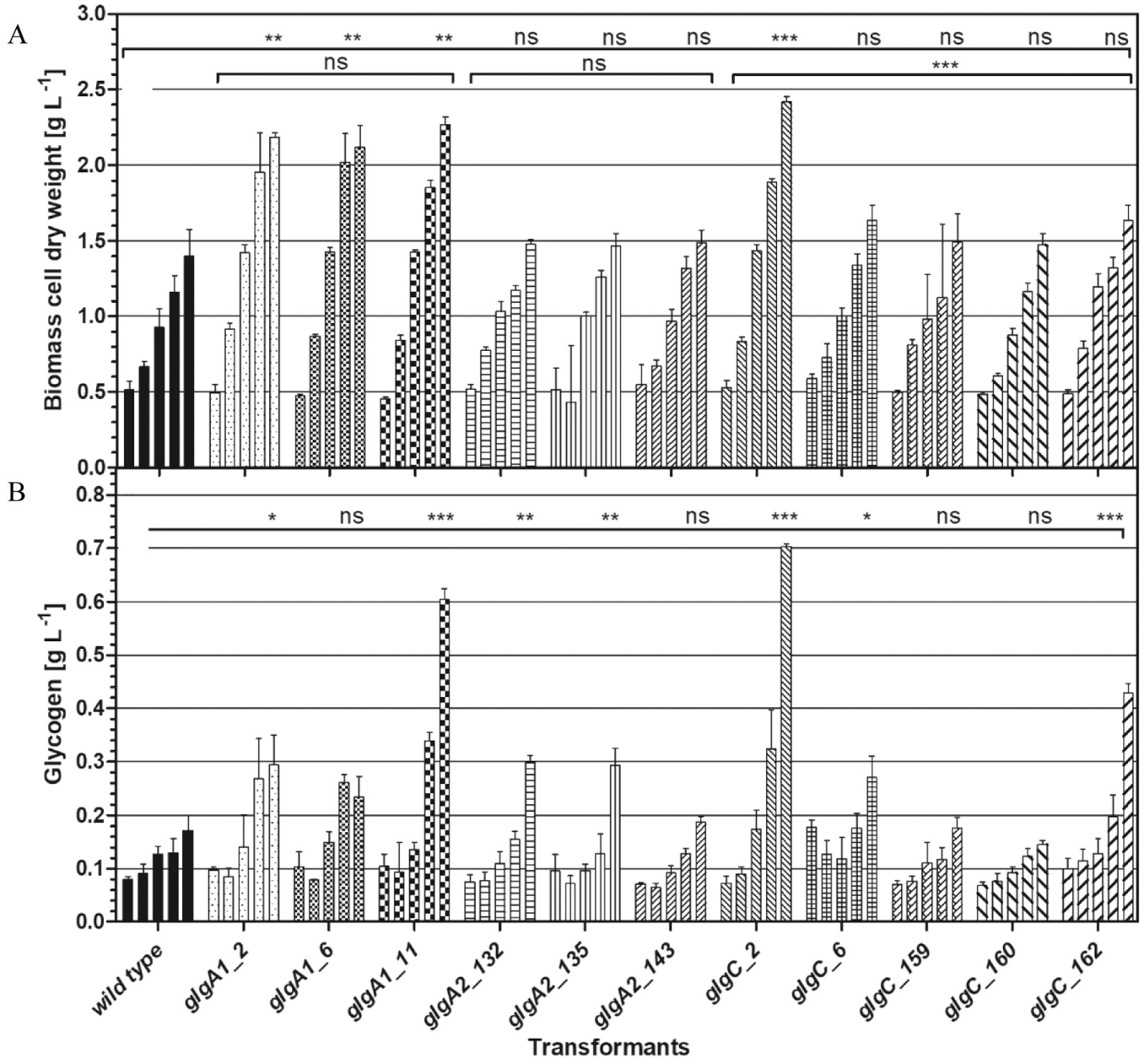

Fig. 4. Determination of dry cell weight and glycogen accumulation. Comparison of the *Synechocystis* sp. PCC6803 wild type strain and its eleven transformants during a 96 h cultivation experiment (starting with sampling immediately after inoculation) in sterile glass columns containing an initial biomass density of 0.5 g L^{-1} in 350 mL BG11 medium supplemented with air and 2% CO_2 at 30 °C and 100 µmol photons m^{-2} s^{-1} regarding their (A) biomass cell dry weight measured daily by gravity measurements of dried culture samples on a filter paper and (B) glycogen content measured daily by liquid chromatography after glycogen monomerization by acidic hydrolysis. The five bars in each investigated strain represent the sampling times 0 h, 24 h, 48 h, 72 h, 96 h (from left to right). Bracket over *glgA1*-, *glgA2*- or *glgC*-transformants: significance within this group of transformants. Brackets over all investigated strains: significances of single transformants compared to the wild type. Significance: ns - not significant ($P > 0.05$); *- significant ($P = 0.01–0.05$); ** - very significant ($P = 0.001–0.01$); *** extremely significant ($P < 0.001$). The transformants were cultivated in triplicate.

*glgC*_162, *glgA1*_2, *glgA1*_6 and *glgA1*_11 was investigated. The goal was to find the transformants that reached the highest cell densities. These transformants would also have the highest energy and precursor supply for subsequent production as e.g. in a two-stage production process.

All tested transformants achieved a higher DCW compared to the WT (1.4 g L^{-1}) after 96 h of cultivation (Fig. 4A). Whereas the *glgA1*_2, *glgA1*_6, *glgA1*_11 and *glgC*_2 transformants grew significantly better compared to the WT, no differences within transformants carrying the same gene were expected, as the integrated expression construct was identical. The transformants overexpressing *glgA1* reached a similar DCW between 2.12 and 2.27 g L^{-1} and an avg. STY_{DCW} of 18.7 mg L^{-1} d^{-1} (shown in Table 1). The same is shown for the *glgA2*-transformants (between 1.46 and 1.49 g L^{-1}). In contrast, the transformant *glgC*_2 produced up to 60% more biomass (2.42 g L^{-1}) compared to the other *glgC* overexpressing transformants (between 1.47 and 1.63 g L^{-1}). This *glgC*_2 is the only transformant out of five investigated *glgC* strains, which shows significant changes in the growth behavior compared to the WT.

The comparison of the transformants harboring different genes also showed that *glgC*_2 produced the highest DCW compared to the *glgA1*- and *glgA2*-transformants after 96 h (Fig. 4A). However, considering the max. and avg. STY_{DCW}, the three *glgA1* transformants showed a slightly

Table 1
Dry cell weight and glycogen accumulation of the *Synechocystis* sp. PCC6803 wild type and transformants.

Culture	% (g L^{-1} glycogen per g L^{-1} DCW[1])[3]	Space-time yield of DCW[1,3]		Space-time yield of glycogen[3]	
		mg L^{-1} h^{-1} (max.)[1,2]	mg L^{-1} h^{-1} (avg.)[1]	mg L^{-1} h^{-1} (max.)[1,2]	mg L^{-1} h^{-1} (avg.)[1]
WT[1]	12.2 ± 0.5	9.2[d] ± 1.1	8.3 ± 1.1	0.99[b,d] ± 0.2	0.78 ± 0.2
*glgA1*_2	13.5 ± 2.2	20.3[c] ± 3.6	18.7 ± 1.2	2.38[c] ± 0.8	1.22 ± 1.1
*glgA1*_6	11.0 ± 0.9	21.4[c] ± 1.3	18.7 ± 2.4	2.19[c] ± 0.5	0.89 ± 1.2
*glgA1*_11	25.0 ± 0.7	20.3[b] ± 0.2	18.7 ± 1.6	5.20[d] ± 0.1	2.15 ± 2.2
*glgA2*_132	20.2 ± 0.6	10.7[a,b] ± 1.6	10.1 ± 0.7	2.33[d] ± 0.1	1.07 ± 0.8
*glgA2*_135	20.1 ± 1.8	10.4[c] ± 1.4	6.7 ± 5.9	2.06[d] ± 0.4	0.39 ± 1.1
*glgA2*_143	12.6 ± 0.2	10.7[c] ± 2.4	8.5 ± 2.2	1.20[d] ± 0.1	0.53 ± 0.5
*glgC*_2	29.1 ± 0.5	19.7[d] ± 0.3	17.6 ± 2.8	6.57[d] ± 0.1	3.22 ± 2.2
*glgC*_6	21.2 ± 3.9	10.9[d] ± 0.7	9.0 ± 2.0	0.96[d] ± 0.5	-0.62 ± 1.2
*glgC*_159	11.8 ± 0.13	13.0[a] ± 1.3	10.5 ± 1.6	1.10[d] ± 0.2	0.71 ± 0.4
*glgC*_160	9.9 ± 0.8	10.3[d] ± 0.5	8.2 ± 2.0	0.80[d] ± 0.1	0.59 ± 0.2
*glgC*_162	26.3 ± 1.9	14.6[b] ± 1.8	11.3 ± 1.2	3.43[d] ± 0.3	1.50 ± 1.2

[1] Abbreviation: max. - maximal, avg. – average, DCW – dry cell weight, WT – wild type.
[2] The maximal production refers to the time points a - 24 h, b – 48 h, c – 72 h, d – 96 h.
[3] Standard deviation is given for each value, n = 3.

increased growth compared to *glgC*_2 (Table 1). The max. STY_{DCW} of *glgC*_2 was significantly lower compared to all three *glgA1* transformants. All three *glgA2*-transformants revealed the slowest growth, being only slightly better compared to the WT. In addition, the *glgA2* transformants were the only ones which died during the long-term cultivation trials. This suggests that the overexpression of *glgA2* does not have a long-term beneficial growth effect on *Synechocystis* in contrast to the other two genes. The overexpression of *glgA1* resulted in an up to 62% biomass increase for *glgA1*_11 compared to the WT. Koch et al. (2019) showed that *Synechocystis* was unable to recover from nitrogen starvation after the *glgA1*-KO, while after the *glgA2*-KO recovery occurred to levels similar to the WT. Together with the results described, this suggests that *glgA1* may have a higher impact on the growth behavior of *Synechocystis* than *glgA2*. Besides, the glycogen synthases, *glgC*_2 showed 73% more DCW than the WT in the scope of this work. Thus, the *glgC*_2 and all *glgA1*-transformants are the most promising transformants to yield a high cell number.

3.6. Determination of the glycogen accumulation

In order to determine the glycogen content of the transformants, culture samples were collected daily and analyzed by LC after acid hydrolysis. The total glycogen accumulation (Fig. 4B) was evaluated in g L^{-1}, as well as its relation to the gained biomass in g L^{-1} glycogen per g L^{-1} DCW and the avg. and max. $STY_{glycogen}$ (Table 1) of the 96 h cultivation in g L^{-1} d^{-1}. The following comparisons of the total glycogen content refer to the 96-h time point of the cultivation and the max. $STY_{glycogen}$. Significant differences in glycogen accumulation were observed among transformants overexpressing the same gene compared to DCW. The transformant *glgA2*_143, which showed slightly increased DCW after 96 h produced approximately 40% less glycogen (0.19 g L^{-1}) than *glgA2*_132 and *glgA2*_135 (0.3 g L^{-1}, 0.29 g L^{-1}). Similar results are obtained concerning the avg. $STY_{glycogen}$ of 1.20 mg L^{-1} h^{-1}, 2.33 mg L^{-1} h^{-1} and 2.06 mg L^{-1} h^{-1}, respectively. The transformant *glgA1*_11 showed over 60% more glycogen accumulation (0.60 g L^{-1}, 5.20 mg L^{-1} h^{-1}) than the other two transformants, which had a similar accumulation and avg. $STY_{glycogen}$ (*glgA1*_2: 0.29 g L^{-1}, 2.38 mg L^{-1} h^{-1} and *glgA1*_6: 0.23 g L^{-1}, 2.19 mg L^{-1} h^{-1}). The transformants *glgC*_159, *glgC*_160 and *glgC*_6 revealed the lowest glycogen accumulation (0.18 g L^{-1}, 0.16 g L^{-1} and 0.27 g L^{-1} respectively), similar to the *glgA2*_143 and the WT (0.17 g L^{-1}, 0.99 mg L^{-1} h^{-1}). The third most productive strain regarding glycogen productivity was *glgC*_162, producing 0.43 g glycogen L^{-1} after 96 h (avg. $STY_{glycogen}$: 3.43 mg L^{-1} h^{-1}). This transformant is promising concerning glycogen production, but its DCW did not differ significantly from the WT. *glgC*_2 showed the highest DCW as well as the highest glycogen accumulation (0.7 g L^{-1}) resulting in the highest avg. $STY_{glycogen}$ (6.57 mg L^{-1} h^{-1}) of all tested transformants and more than a 4-fold glycogen accumulation increase compared to the WT.

In these trials, no *glgA2*-transformant showed as much an increase in biomass and glycogen production as the two best producing transformants *glgA1*_11 and *glgC*_2. It suggests that the overexpression of the *glgA2* gene in *Synechocystis* did not markedly affect the DCW as well as the glycogen accumulation compared to the overexpression of the other two genes. The C-terminal protein tag could influence the activity on the GlgA2 since the protein was synthesized but a significant difference to the wild type in biomass and glycogen was not detected. The transformants *glgA1*_11 and *glgC_2* reached about 1.6- and 1.7-fold increased DCW and 3.5- and 4-fold increased glycogen accumulation, respectively, compared to the WT after 96 h of cultivation. As *glgA1*_11 shows higher biomass and *glgC*_2 shows higher glycogen production, both are promising strains for the use in further applications like increased initial cell density or precursor availability for e.g. a two-stage production process within one cell.

According to the literature, the glycogen accumulation in *Synechocystis* can be enhanced by nitrogen and phosphorus limitation (Yoo et al., 2007). However, the glycogen productions presented here were reached without any nutrient limitations during cultivation and still show a significant glycogen increase compared to the WT. If a high number of vital cells for subsequent production steps will be needed, nutrient deprivation would be disadvantageous as the cells are stressed and growth might be limited. Thus, the limitation step would prolong the cultivation process resulting in lower glycogen productivity and decreased economic efficiency.

Within this study, identical expression vectors (backbone, P_{cpc560}, Spe^R) were used, therefore, this suggests the glycogen accumulation differs as a result of the integrated gene. It suggests that the effect of the expression vector is gene dependent. As a result, it was important to screen multiple transformants to find the most productive one as the individual transformants demonstrated significant variability in DCW as well as glycogen accumulation. Further studies on the heterogeneity of genome sequences and varying mRNA levels would be necessary for a better understanding of the different behaviors since all transformants should have been modified at the same genome location. Moreover, the role of the *glgA2* gene in glycogen synthesis seems to be minor compared to *glgA1*, since the overexpression only leads to a slight increase in the glycogen production in all investigated transformants. This result contradicts the statement of Yoo et al. (2014) that the two synthases are redundant to one another as the KO of one synthase did not lead to a change in glycogen accumulation. In the scope of this work, an overexpression with a subsequent overproduction of glycogen could only be found with a *glgA1* transformant compared to the WT. Since Yoo et al. (2014) used a heterotrophic cultivation strategy and a photoautotrophic strategy was used in the presented work, these two studies can hardly be compared directly, and the influence of the cultivation parameters need to be investigated further.

3.7. Correlation of the used tools and glycogen accumulation

In this study, the highest amount of glycogen could be achieved by fewer genetic modifications and using standard cultivation conditions compared to the most productive strains of glycogen published so far (Velmurugan and Incharoensakdi, 2018). The most productive strain *glgC*_2 of this study, carrying an overexpression of the gene *glgC*, produced 29.1% glycogen (g L^{-1} per g DCW L^{-1}) in *Synechocystis* after 96 h of cultivation (350 mL BG11 medium, 2% CO_2, 30 °C, 100 µmol photons m^{-2} s^{-1}, $OD_{initial}$ = 0.5 g L^{-1}). This resulted in the max. STY_{DCW} of about 19.7 mg L^{-1} h^{-1}, which refers to 6.57 mg glycogen L^{-1} h^{-1}. The highest glycogen production in a modified *Synechocystis* sp. PCC6803 published so far, achieved by Velmurugan and Incharoensakdi (2018), was 54.0% (g L^{-1} per g DCW L^{-1}) after 20 days (100 mL BG11 medium, 3 mmol L^{-1} H_2O_2, atmospheric CO_2, 28 °C, $OD_{initial}$ = 0.06). It was achieved by overexpressing the *glgA1* and *glgC* genes simultaneously under the control of the P_{psbA2} promoter, the KO of the PHB pathway by *phaA* deletion and the use of the integration site harbored by the pEERM4 plasmid. Their results showed a 2.24-fold increased glycogen accumulation compared to the WT (24.1%) (Velmurugan and Incharoensakdi, 2018). In contrast, a 2.39-fold increase to the WT production (12.2%) could be achieved in this study by only overexpressing *glgC* and without any KO of competitive pathways or the application of nutrient limitation.

The cultivation of the *Synechocystis* strain harboring only the overexpressed *glgC* gene resulted in 29.7% after 20-days of cultivation (14.9 mg glycogen g^{-1} DCW d^{-1}) (Velmurugan and Incharoensakdi, 2018). The glycogen per DCW and time of *glgC*_2, generated in this study, is approximately 4.5-fold higher. Moreover, *glgC*_2 showed 1.16-fold more glycogen after 94 h (4 days) than the best-producing strain of Velmurugan and Incharoensakdi (2018) after 10 days of cultivation (approximately 25%) both without nutrient limitation. The data obtained during the day 10 was used for comparison, as the culture of Velmurugan and Incharoensakdi (2018) had the same DCW on Day 5 as the initial DCW (0.5 g L^{-1}) used in this study, resulting in the same process time for comparison. This suggests that the combination of NSC1 with the P_{cpc560} promoter driving the gene expression of *glgC* is a suitable expression system for increased glycogen production in *Synechocystis*. Furthermore, it shows that the use of promising molecular biology tools leads to the desired production increase. So far, no detailed production optimizations like the determination of optimal light intensities and culture densities for optimal promoter induction have been carried out. Moreover, the screening of additional transformants showing high protein production or further additional genomic optimizations like the KO of competing pathways (e.g. PHB) as well as the co-overexpression of the genes *glgC* and *glgA1*, leading to increased glycogen accumulation, are to be performed.

Furthermore, the integration of genes from foreign synthetic pathways into these transformants is a possible further approach. The integration of genes like those for the bioethanol synthesis regulated by an inducible promoter, could successfully implement a two-stage production process. In the first step, a high cell density with accumulated glycogen (e.g. transformant *glgC*_2) could be generated. The glycogen could then in turn be used as a precursor for bioethanol synthesis after induction of a promoter regulating a gene required for the second step. As increased amounts of precursors would be available the resulting bioethanol yield may be elevated.

4. Conclusion

The glycogen production process in *Synechocystis* was improved by the overexpression of the three main genes (*glgA1, glgA2, glgC*) responsible for glycogen synthesis, using the strong promoter P_{cpc560} and the neutral cloning site NSC1. Both of these genetic elements are published to be either strong enhancers for gene expression or long-term stable genome integration (Ng et al., 2015; Zhou et al., 2014). The complete genome integration, protein synthesis, biomass and glycogen production were determined to identify the most promising transformants. A long-term stable transgene integration was shown as well as the impact on genomic integration causing increased DCW and glycogen accumulation. The overexpression of *glgC* and *glgA1* resulted in the generation of the transformants *glgC*_2 and *glgA1*_11 with significantly increased DCW and glycogen accumulation compared to the WT. In contrast, the overexpression of *glgA2* hardly affected either the DCW or the glycogen accumulation of *Synechocystis* as much as the overexpression of the other two genes.

The two transformants *glgC*_2 and *glgA1*_11 are promising candidates for successfully enhancing the production of toxic compounds like bioethanol in a two-stage production strategy. By integrating the genes responsible for ethanol production into the genome of *glgC*_2 and *glgA1*_11, the cell density as well as the precursor and energy supply could be greatly increased in these pre-cultures. This in turn would allow for a clearly more efficient production of bioethanol after the induction of an inducible promotor in a second processing step, yielding bioethanol concentrations that have yet to be reached using *Synechocystis*.

CRediT authorship contribution statement

Sandra Mittermair: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Gergely Lakatos:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Writing - Review. **Cecilia Nicoletti:** Investigation, Writing – original draft, Writing - Review. **Karolína Ranglová:** Methodology, Investigation, Validation, Writing - Review. **João Câmara Manoel:** Investigation, Writing - Review. **Tomáš Grivalský:** Investigation, Validation Writing - Review. **Kozhan Daniyar Malikuly:** Investigation; **Jiří Masojídek:** Resources, Writing - Review, Supervision, Funding acquisition; **Juliane Richter:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

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.

Data availability

The data that supports the findings of this study are available in the supplementary material of this article.

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Author contributions

S.M., **G.L.**, **J.R.** and **J.M.** contributed to the conception and design of the study. **S.M.**, **G.L.**, **C.N.**, **K.R.**, **J.C.M.**, **T.G.**, **K. D. M.** and **J.R.** performed the research. **S.M.**, **G.L.**, **K.R.** and **T.G.** analyzed and interpreted the data. **S.M.**, **G.L.**, **C.N.** and **J.R.** prepared the manuscript with input from the other authors. All authors commented on the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

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