Algal Research 37 (2019) 57–63

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

# Metabolic engineering of cyanobacteria for photoautotrophic production of heparosan, a pharmaceutical precursor of heparin

Aditya Sarnaik[a,b,1], Mary H. Abernathy[b,1], Xiaorui Han[c], Yilan Ouyang[c], Ke Xia[c], Yin Chen[c], Brady Cress[d], Fuming Zhang[d], Arvind Lali[a], Reena Pandit[a,*], Robert J. Linhardt[c], Yinjie J. Tang[b,*], Mattheos A.G. Koffas[d,e,**]

[a] DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai 400019, Maharashtra, India
[b] Department of Energy, Environmental and Chemical Engineering, Washington University in St. Louis, One Brookings Dr., St. Louis, MO 63130, USA
[c] Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, USA
[d] Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, USA
[e] Department of Biological Sciences, Rensselaer Polytechnic Institute, 110 8th St., Troy, NY 12180, USA.

ARTICLE INFO

*Keywords:*
Glycosaminoglycans
Cyanobacteria
Heparin
Photoautotrophic production
Polysaccharides
*Synechococcus*

ABSTRACT

Heparosan is an unsulfated polysaccharide potentially important for its wide range of cosmetic and pharmaceutical applications, particularly as the precursor for the extensively used anticoagulant, heparin. Generally sourced from animals, commercially available heparin may encounter various immunological and contamination risks. Thus, safe and sustainable microbial platforms could serve as an alternative heparin source. *Synechococcus*, due to their fast photoautotrophic growth, strong sugar phosphate metabolisms and generally regarded as safe (GRAS) nature, may serve as photo-biorefineries for manufacturing heparosan. In this study, we have synthesized an integrative plasmid *pUPm48* for cloning *galU* and *PmHS2* genes in *Synechococcus elongatus* PCC 7942. The engineered recombinants (*pgp7942*) exhibited significant production of heparosan under different culture conditions, where the products were present in both supernatant and cell biomass. The maximum yield of 0.7 ± 0.2 μg/g-DCW (dry cell weight) and a titer of 2.8 ± 0.3 μg/L was achieved by *pgp7942* under shake flask and continuous light conditions. Large scale plastic-bag cultures with natural diurnal light exhibited heparosan production of 0.5 μg/g-DCW with a titer of 0.44 μg/L. The analysis also found PCC 7942 encodes a promiscuous uridyltransferase for UDP-glucose synthesis and naturally produces multiple glycosaminoglycans including chondroitin sulfate (CS). This study demonstrates for the first-time cyanobacteria as a promising photoautotrophic refinery for producing a high-value polysaccharide commonly from animals.

## 1. Introduction

Glycosaminoglycans (GAGs) are a class of saccharides consisting of repeating units of amino sugars and uronic acids and have increasingly enticed research and commercial interests due to their wide range of physiological functions [1–5]. These GAGs, heparan sulfate, chondroitin sulfate, and hylauronic acid (Fig. 1), are naturally produced across a range of organisms and play key roles in moisture retention, cell adhesion and proliferation. Currently GAGs are mainly extracted from food animal tissues (approximately 100 mg per kg of tissue) [6] and are widely used in pharmaceutical and cosmetic applications. However, immunological reactions and interspecies disease transfers are a major problem, and a contaminated global supply of heparin (an important anticoagulant) in 2007–8 resulted in 100 deaths [2]. *De novo* chemical synthesis of heparin has been attempted and one ultra-low molecular weight heparin (< 1.5 kDa, *fondaparinux*) has been commercially successful, but yields are low and biological activity is challenging to replicate as a result of polymerization and complex sulfation [7].

The precursor of heparin and heparan sulfates (HS) [8,9] is heparosan (HS-0S), an unsulfated polysaccharide consisting of a linear copolymer of repeating units α-1,4 linked D-glucosamine (*GlcNAc*) and β-1,4-D-glucuronic acid (*GlcUA*) [10]. Heparosan has also been used to enhance the efficacy of protein-based therapeutics by forming drug

* Corresponding authors.
** Correspondence to: M.A.G. Koffas, Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, USA.
*E-mail addresses:* ra.pandit@ictmumbai.edu.in (R. Pandit), yinjie.tang@wustl.edu (Y.J. Tang), koffam@rpi.edu (M.A.G. Koffas).
[1] Contributed equally as co-authors.

https://doi.org/10.1016/j.algal.2018.11.010
Received 7 June 2018; Received in revised form 10 November 2018; Accepted 22 November 2018

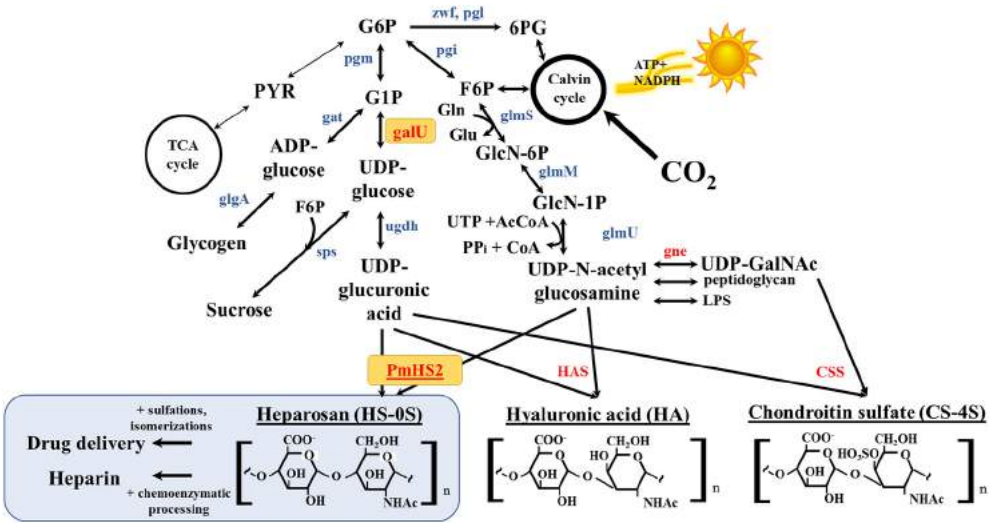


**Fig. 1.** Schematic featuring the biosynthetic production pathway of glycosaminoglycans in *Synechococcus* PCC 7942, specifically the expression of heparosan and its downstream applications. Genes in red indicate genes that are not annotated in KEGG database. Genes in blue are naturally present in *Synechococcus* 7942. Genes in yellow boxes were heterologously expressed in recombinant *Synechococcus* 7942 for the production of heparosan. 6PG, 6-phosphogluconic acid; AcCoA, acetyl CoA; ADP, adenosine diphosphate; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GalNAc, *N*-acetylgalactosamine, GlcN-1P, glucosamine-1-phosphate; GlcN-6P, glucosamine-6-phosphate; Gln, glutamine; Glu; Glutamic acid; PPi; pyrophosphate, PYR, pyruvate; TCA, tricarboxylic acid; UDP, uridine diphosphate; UTP, uridine triphosphate; (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conjugates and could serve as a replacement for polyethylene glycol coatings for drug delivery applications [11]. In recent years, the biosynthesis of GAGs from microbial production platforms has emerged as an alternative to extraction from animal sources [12,13]. *In vitro* chemoenzymatic synthesis from *in vivo* synthesized heparosan could serve as an effective alternative to animal derived heparin [9,14,15]. As a result, efficient heparosan synthesis becomes the primary step towards heparin production [9]. Heparosan is naturally produced by pathogenic bacteria, such as *E. coli* K5, *Pasteurella multocida*, *Avibacterium paragallinarum* as a part of polysaccharide capsules, which impart virulence to these organisms [9,10]. Heparosan synthesis by genetically modified non-pathogenic *Bacillus subtilis* and *E. coli* BL21 has been successfully achieved by cloning *kfiC* and *kfiA* genes from *E. coli* K5, yet molecular weight and homogeneity were difficult to control ([10,16]. Nevertheless, *Pasteurella multocida* possesses a *PmHS2* gene that encodes for a dual functional heparosan synthase that replaces the two genes, *kfiC* and *kfiA*, [17] and results in smaller-molecular-weight polysaccharide chains [18].

This study aimed at engineering cyanobacteria using *PmHS2* from *P. multocida* (Fig. 2A). Being GRAS (generally recognized as safe), cyanobacteria can serve as photosynthetic platform for production of valuable chemicals [19,20]. Compared to eukaryotic photoautotrophs, cyanobacterial hosts have established genomic data, molecular manipulation tools, low cost of culture mediums, and generally faster growth [21]. Moreover, cyanobacteria possess strong metabolic fluxes through its sugar phosphate pathways and high nucleotide sugar pool sizes [22]. They naturally synthesize complex polysaccharides like glycans for colonization, symbiosis, protection, and food reservation ([23–25]. However, to the best of the authors' knowledge, the production of GAGs with specific disaccharide repeating units has not been previously reported in cyanobacteria. Here, we selected a model cyanobacterium *Synechococcus elongatus* PCC 7942 (PCC 7942) for the photosynthetic synthesis of heparosan to demonstrate their potential as a new chassis for the synthesis of value-added carbohydrates and high-value pharmaceuticals (Fig. 1).

## 2. Materials and methods

### 2.1. Chemicals and reagents

T4 DNA ligase, restriction enzymes, and shrimp alkaline phosphatase were purchased from New England Biolabs. PrimeSTAR Max DNA polymerase (2×) high fidelity PCR master-mix was purchased from *Clontech* (DSS *TaKaRa* Bio India Pvt. Ltd.). *NucleoSpin®* Gel and PCR Clean-up kit was purchased from Macherey-Nagel (*MN*, India). Plasmid extraction Miniprep kit was purchased from *GeneAll* (*AllianzBio*, Mumbai, India).

### 2.2. Microorganisms and culture conditions

Construction and amplification of recombinant plasmids were performed in *E. coli* Top10F (*ThermoFisher Scientific*) cells. Cells were grown at 37 °C in Luria Bertani (LB) broth supplemented with 100 μg/mL spectinomycin (*HiMedia*) [26]. For PCC 7942 cultivation, the seed cultures were grown with continuous illumination of 55 ± 2 μmol/$m^2$/s and 28 ± 0.5 °C temperature. BG-11 medium (supplemented with 30 μg/mL of spectinomycin in case of transformants) was used. Experiments were performed under several laboratory conditions in shake flasks (50 mL culture volumes) at a speed of 250 rpm at atmospheric (0.04% (v/v)) (LC) and 0.2% (v/v) $CO_2$ (HC) at 37 °C and continuous light environments (100 photons μmol/$m^2$/s (LL) and 250 photons μmol/$m^2$/s (HL)). Low nitrogen cultures were prepared with BG-11 medium with 1/5 $NaNO_3$ (0.15 g/L) to promote carbohydrate accumulation, while 30 mM of glycerol was added to cultures for photomixotrophic growth (Fig. 2C). Both cell pellet and supernatant samples were analysed for heparosan. All experiments were performed in duplicates. Cultures were induced with 1 mM of isopropyl β- D-1-thiogalactopyranoside (IPTG) at early exponential growth. Scale-up was performed in an environmental laboratory (EL), a state-of-the-art glass house facility at DBT-ICT Centre for Energy Biosciences, Mumbai, India. In the EL, cells were grown under natural diurnal light (maximum 1200 ± 200 μmol/$m^2$/s, 33 ± 10 °C) in a bubble column plastic bag reactor (air bubbling rate of 60 mL/min) using atmospheric $CO_2$ at a 5 L culture volume. Culture pellet was analysed for heparosan production. Culture was induced with 1 mM of IPTG at early growth and no antibiotic was added during scale-up. Culture growth was monitored by recording optical densities of the cultures at 730 nm using a *UV–Vis* spectrophotometer and measuring the dry cell weight (DCW) of the lyophilized cell pellets.

### 2.3. Cyanobacterial plasmid construction and transformation

The plasmid, *pUPm48*, was constructed using the *pAM2991* vector for transformation of PCC 7942. Initially, the plasmid *pU48* was

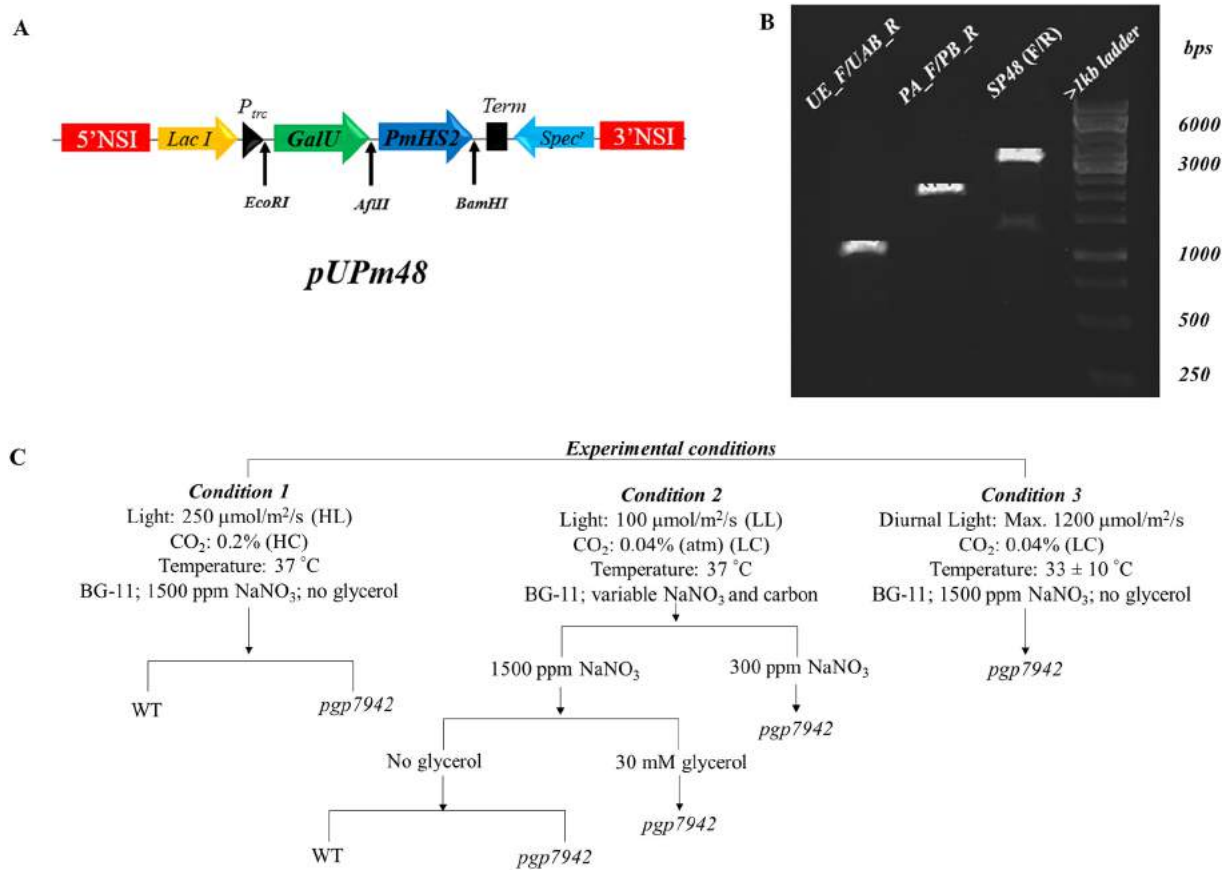


**Fig. 2.** Diagrams of recombinant *Synechococcus* PCC 7942 (*pgp7942*) grown in various conditions for the production of heparosan. (A) Representation of the synthetic operon construct, *pUPm48*, for homologous recombination into NSI of chromosome. (B) PCC 7942 transformants, pgp7942, obtained after complete segregation of the cloned gene were analysed through colony PCR and agarose gel electrophoresis. galU amplicon in lane 1, PmHS2 amplicon in lane 2, entire operon (galU + PmHS2) has been amplified using NSI specific primers in lane 3; followed by > 1 kb DNA ladder in lane 4. Primers used are listed in Table 1. (C) Variable growth conditions to grow pgp7942 and wild-type (WT) PCC 7942. The strains were grown under two distinct shake flask conditions; 250 photons µmol/$m^2$/s (HL) and 0.2% (v/v) $CO_2$ (HC) (condition 1), 100 photons µmol/$m^2$/s (LL) and 0.04% (v/v) atmospheric $CO_2$ (LC) with variations in media composition (condition 2). Pgp7942 cells were scaled up under natural diurnal light conditions with irradiance of maximum 1200 ± 200 µmol/$m^2$/s and temperature 33 ± 10 °C with atmospheric $CO_2$ (0.04%) (condition 3).

**Table 1**
List of primers and sequences used. The underlined sequences are the restriction sites for cloning the genes.

| Primer names | Sequences |
|---|---|
| *UE_F* | 5′-GACTGAATTCATGGCTGCCATTAATACGAA-3′ |
| *UAB_R* | 5′-ACTTGGATCCCTTAAGTTACTTCTTAATGCCCATCTC-3′ |
| *PA_F* | 5′-AAGTCTTAAGATGAAGAGAAAAAAAGAGATG-3′ |
| *PB_R* | 5′-AATCGGATCCTCACAAAAAATAAAAAGGTAAACAG-3′ |
| *SP48_F* | 5′- GTCTTTCGACTGAGCCTTTCG - 3′ |
| *SP48_4* | 5′ - CAGGCAGCCATCGGAAGC - 3′ |

constructed by cloning the *galU* gene amplified from *E. coli* MG1655 (UTP-glucose-1-phosphate uridyltransferase, NC_000913.3) genomic DNA between restriction sites, *EcoRI* and *BamHI* of the *pAM2991* vector (Primers; *UE_F* and *UAB_R*) (Table 1). The restriction *AflII* site was introduced in the construct using the *galU* gene reverse primer (*UAB_R*) for the introduction of another gene into *pU48*. The plasmid *pUPm48* was constructed by cloning the *PmHS2* gene (Heparosan synthase B, *AY292200.1*) amplified from *Pasteurella multocida* genomic DNA between the restriction sites *AflII* and *BamHI* sites of the *pR48* vector (Primers; *PA_F* and *PB_R*) (Table 1) (Fig. 2A). PCC 7942 transformants (*pgp7942*) were developed based on homologous recombination strategy using *pUPm48* through natural transformation. The transformation protocol was followed as demonstrated by Clerico et al [27]. Colonies obtained on plates were passaged at least four times to get stable transformants [26]. Gene integration in cyanobacterial genome was confirmed by colony PCR using neutral site primers *SP48_F* and *SP48_R* (Table 1) (Fig. 2B). Agarose gel electrophoresis of the colony PCR samples show bands corresponding to 1 kbps (lane 1, *galU* gene), 2.2 kbps (lane 2, *PmHS2* gene) and 3.5 kbps (lane 3, neutral site primers).

### 2.4. Analysis of heparosan production using LC-MS

#### 2.4.1. Materials for digestion of samples and LC-MS analysis

Unsaturated disaccharide standards of CS (ΔUA-GalNAc; ΔUA-GalNAc4S; ΔUA-GalNAc6S; ΔUA2S-GalNAc; ΔUA2S-Gal-NAc4S; ΔUA2S-GalNAc6S; ΔUA-GalNAc4S6S; ΔUA2S-GalNAc4S6S), unsaturated disaccharide standards of HS (ΔUA-GlcNAc; ΔUA-GlcNS; ΔUA-GlcNAc6S; ΔUA2S-GlcNAc; ΔUA2S-GlcNS; ΔUA-GlcNS6S; ΔUA2S-GlcNAc6S; ΔUA2S-GlcNS6S), and unsaturated disaccharide standard of HA (ΔUA-GlcNAc), where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, were purchased from Iduron (UK). Actinase E was obtained from Kaken Biochemicals (Japan). Various polysaccharide lyases were obtained for digestions. Chondroitin lyase ABC from *Proteus vulgaris* was cloned and expressed in *E. coli* and purified in the Linhardt lab. Recombinant Flavobacterial heparin lyases I, II, and III were also expressed and purified by the Linhardt lab using *E. coli* strains provided by Jian Liu (College of Pharmacy, University of North Carolina). 2-

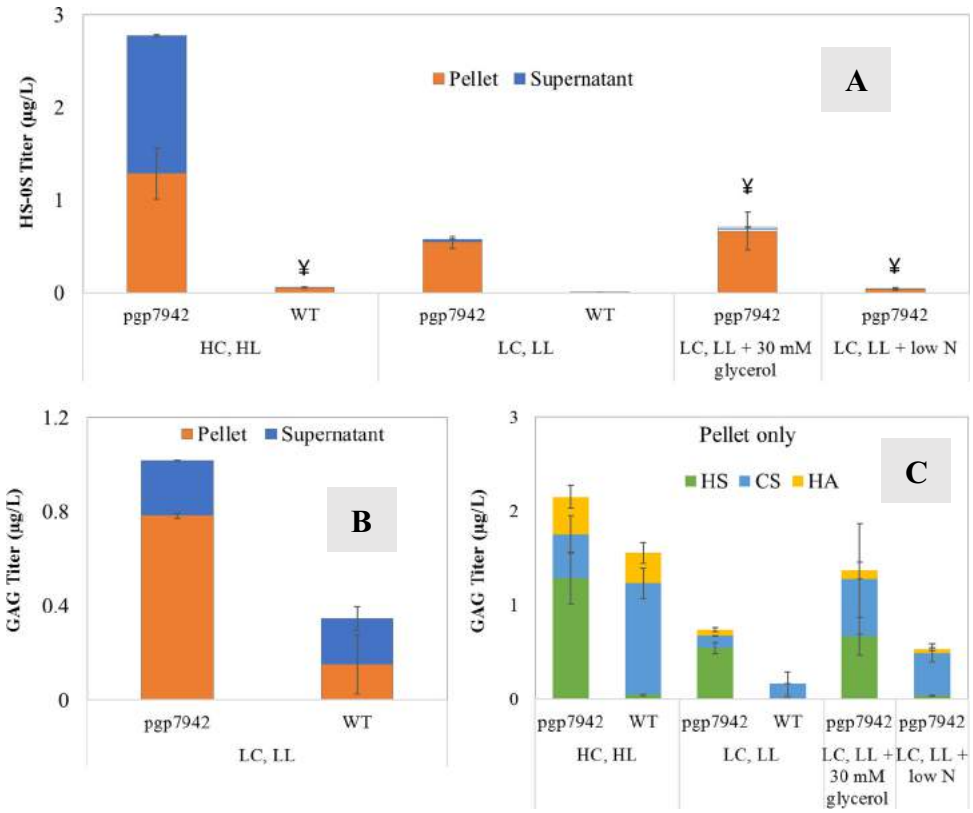


**Fig. 3.** Heparosan producing strain, *pgp7942*, compared to *Synechococcus* 7942 WT under continuous light shake flask conditions. Shaking flask conditions vary in light from 100 photons µmol/$m^2$/s (LL) to 250 photons µmol/$m^2$/s (HL) and atmospheric $CO_2$ (0.04% (v/v)) (LC) to 0.2% (v/v) $CO_2$ (HC), but are held at a temperature of 37 °C, induced with 1 mM of IPTG, and have an rpm of 250 for 50 mL culture volumes. Low N indicates BG-11 was used with 1/5 of the standard amount of nitrogen typically used. Standard deviations are based off n = 2. (A) Titer of heparosan (HS-0S) in cell pellet and extracellular media. ¥ Conditions that the HS-0S in supernatant was not well analysed due to innate low concentrations and high measurement noises. (B) Titer of total glycosaminoglycans (GAGs) in both cell pellet and extracellular media. (C) Break down of GAG titer in the cell pellet only as heparin sulfate (HS), chondroitin sulfate (CS) and hyaluronic acid (HA).

Aminoacridone (AMAC) and sodium cyanoborohydride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of HPLC grade. Vivapure Q Mini H strong anion exchange spin columns were from Sartoriou Stedim Biotech (Bohemia, NY, USA).

### 2.4.2. GAG extraction, sample desalination and digestion

Lyophilized pellets were re-suspended with 15 mL of $ddH_2O$, then sonicated for 10 min in an ice bath using a Misonix Sonicator 3000 (600 watts of energy deliver) with microtip at 40% magnitude, followed by centrifugation at 3220 relative centrifugal force (RCF) for 10 min. The supernatant was then collected and freeze-dried. Dried supernatant and extracted pelleted samples were re-dissolved in 400 µL $ddH_2O$ and desalted by passing through a 3 kDa molecule weight cut-off spin column. The casing tubes were replaced before 200 µL of digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0) was added to the filter unit. Recombinant heparin lyase I, II, III (pH optima 7.0–7.5) and recombinant chondroitin lyase ABC (10 mU each, pH optimum 7.4) were added to each sample and mixed well. The samples were all placed at 37 °C for 12 h, after which enzymatic digestion was terminated by removing the enzymes by centrifugation. The filter unit was washed twice with 300 µL distilled water and the filtrates containing the disaccharide products were dried *via* vacuum centrifuge.

### 2.4.3. 2-Aminoacridone (AMAC) labelling and LC-MS analysis

The dried samples were AMAC-labeled by adding 10 µL of 0.1 M AMAC in DMSO/acetic acid (17:3 (v/v)) incubating at room temperature for 10 min, followed by adding 10 µL of 1 M aqueous sodium cyanoborohydride and incubating for 1 h at 45 °C. A mixture containing all 17-disaccharide standards prepared at 0.5 ng/µL was similarly AMAC-labeled and used for each run as an external standard. After the AMAC-labeling reaction, the samples were centrifuged and each supernatant was recovered for LC-MS analysis. LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 µm, 3.0 × 50 mm) column. Mobile phase A was 50 mM ammonium acetate aqueous solution, and the mobile phase B was 100% methanol. The mobile phase passed through the column at a flow rate of 300 µL/min. The gradient was 0–10 min, 5–45% B; 10–10.2 min, 45–100% B; 10.2–14 min, 100% B; 14–22 min, 100–5% B. A triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA) was used a detector in multiple reaction monitoring (MRM) mode. The MS parameters were set at negative ionization mode with a spray voltage of 3000 V, a vaporizer temperature of 300 °C, and a capillary temperature of 270 °C.

## 3. Results and discussion

Controlled polymerization of heparosan is an important prerequisite to obtain functionally active molecule. Among various natural producers of heparosan, mammals use an active hetero-complex of glycosyltransferases *EXT1* and *EXT2* yielding a polymer of on average 170 kDa, whereas *E. coli* K5 synthesizes a polymer of 10–20 kDa using synchronous activity of two enzymes *KfiA* (glucosaminyl transferase) and *KfiC* (glucuronyl transferase). On the contrary, *Pasteurella* expresses a dual functional glycosyltransferase *PmHS1* and its cryptic homolog *PmHS2*. Functionally, *PmHS1* has higher affinity towards short oligosaccharides while *PmHS2* exhibits more affinity towards UDP-sugars which are limiting precursor molecules [2]. The gene, *PmHS2*, is a glycosyltransferase responsible for the step-wise addition of UDP-glucuronic acid (UDP-GlcUA) and UDP-N acetyl glucosamine (UDP-GlcNAc) for heparosan polymerization [17]. Based on these reports, the heparosan synthase, *PmHS2* was selected for expression of HS-0S in cyanobacteria. (Fig. 1).

Being GRAS organisms, cyanobacteria are known to be efficient producers of complex polysaccharides that can be extended to pharmaceuticals [28]. However, natural production of heparosan or specific GAGs for biotechnology applications has not yet reported in cyanobacteria ([23,24]. Therefore, cyanobacterium PCC 7942 was explored as the host for production of heparosan. Successful functionality of the *PmHS2* gene product in cyanobacteria requires a large UDP-glucuronic

**Table 2**
Summary of heparosan production in PCC 7942, WT and engineered 7942 (*pgp7942*). Highest heparosan and biomass productivity in the cell pellet are reported for each condition. Standard deviation is based off duplicates. All experiments conducted at 33 °C or 37 °C. *Experiment carried out under natural diurnal conditions in environmental laboratory.

| Strain | Condition description | Carbon source | Light | Heparosan Productivity (μg/L/day) (n = 2) | Biomass productivity (at mid-log phase) (g/L/day) |
|---|---|---|---|---|---|
| *pgp7942* | Shaking flask, HC/HL | 0.2% $CO_2$ | 250 μmol/$m^2$/s (continuous) | 0.21 ± 0.03 | 0.59 ± 0.01 |
| WT | Shaking flask, HC/HL | 0.2% $CO_2$ | 250 μmol/$m^2$/s (continuous) | < 0.01 | 0.57 ± 0.02 |
| *pgp7942* | Shaking flask, LC/LL | 0.04% $CO_2$ (atmospheric) + 30 mM glycerol | 100 μmol/$m^2$/s (continuous) | 0.07 ± 0.03 | 0.35 ± 0.11 |
| *pgp7942* | Shaking flask, LC/LL | 0.04% $CO_2$ (atmospheric) | 100 μmol/$m^2$/s (continuous) | 0.06 ± 0.00 | 0.44 ± 0.01 |
| *pgp7942* | Natural scale-up* | 0.04% $CO_2$ (atmospheric) | Natural diurnal light | 0.09 | 0.25 |

acid pool (Fig. 1), mitigated by expression of *galU* (UDP-glucose pyrophosphorylase). Thus, for HS-0S production in cyanobacteria, the vector construct, *pUPm48*, with the genes *galU* and *PmHS2* under the $P_{trc}$ promoter (Fig. 2A) was successfully integrated into the PCC 7942 genome to create the transformants, *pgp7942* (Fig. 2B).

UDP-glucuronic acid is one of the essential monomeric precursors and is typically synthesized from UDP-glucose. While many cyanobacteria do not have an annotated UDP-glucose pyrophosphorylase that catalyzes the conversion of glucose-1-phosphate to UDP-glucose, some cyanobacteria encode other enzymes relevant to UDP-glucose production and consumption. For example, *Synechocystis* sp. PCC 6803 (PCC 6803) possesses a gene *CugP* (Uniprot ID CUGP_SYNY3) that encodes for UTP-glucose-1-phosphate uridyltransferase, which synthesizes UDP-glucose, same as *galU* [29]. Protein BLAST (*BLASTp*) of this non-*galU* type uridyltransferase exhibited 99% sequence similarity with mannose-1-phosphate guanyltransferase (BAM54903.1) from PCC 6803 (*SI* Fig. 1). Its *NTP_transferase* functional domain belonging to *Glyco_tranf_GTA_type* superfamily was verified from Conserved Domain database at NCBI (domain architecture ID 11440233). *BLASTp* of this domain displayed 79% sequence homology with mannose-1-phosphate guanyltransferase (ABB58003.1, gene: SYNPCC7942_RS10005) from PCC 7942 (*SI* Fig. 2). We hypothesize that the gene RS10005 encodes a promiscuous enzyme that can also synthesize UDP-glucose in PCC 7942. This bi-specificity of mannose-1-phosphate guanyltransferase (gene *RS10005* from PCC 7942) requires further functional annotation. The heterologous gene, *galU*, assists in UDP-glucose formation for a sufficient pool of UDP-glucuronic acid for heparosan production than native RS10005 alone.

Cyanobacteria tend to synthesize polysaccharides under various physical and chemical stress conditions [23]. Therefore, to improve heparosan (HS-0S) production from *pgp7942*, cells were grown under different environmental conditions (Fig. 2C), such as high $CO_2$ (HC), photomixotrophic and nitrogen limitations conditions. The strain *pgp7942* produces significantly higher yields of HS-0S under regular nitrate concentrations than the WT (Fig. 3A) with the highest titer 2779 ± 285 ng/L and a productivity of 278 ± 29 ng/L/day under HL and HC. Glycerol was added to the medium in LC, LL conditions, but did not result in significant improvement of HS-0S production in *pgp7942* (Fig. 3A), with titers of 0.7 ± 0.2 μg/L and 0.6 ± 0.1 μg/L, with and without glycerol respectively under LC, LL conditions. Cultures when grown in BG-11 with low nitrate concentration (300 ppm) exhibited the smallest HS-0S titer in *pgp7942* and significantly impacted HS-0S and GAG titers. This observation contrasts to cyanobacterial glycogen or polyhydroxybutyrate (PHB) accumulation under nitrogen starvation as seen in other studies [30]. The deleterious effect can be explained by a depletion of building blocks for product synthesis, such as glutamine, and a depletion of light harvesting proteins, such as phycobilisomes, during nitrogen limitation [31].

LC-MS analysis showed that all PCC 7942 cultures synthesized various types of GAGs and that heparosan, in addition to other GAGs, was secreted into the supernatant (Fig. 3B). Cyanobacteria are known to produce an extracellular matrix (ECM) to serve as protection from the outside environment. The ECM is composed of complex heteropolysaccharides assembled and exported through multiple pathways and then are attached to the cell surface or freed into the surrounding environment as released polysaccharides [32]. According to phylogenomic analyses, *Synechococcus* has been found to possess the least amount of proteins serving the ECM due to evolutionary loss, which may reduce cross-talk and competition for substrates compared to other cyanobacteria species [33]. Wild-type *Synechococcus* was found to synthesize chondroitin sulfate (CS) (1.2 ± 0.2 μg/L after 120 h under HC, HL conditions) and hyaluronic acid (HA) (0.3 ± 0.1 μg/L after 120 h under HC, HL conditions). The transformant, *pgp7942*, produced similar amounts of HA at 0.4 ± 0.1 μg/L after 120 h under HC, HL conditions, but less CS at 0.5 ± 0.2 μg/L, indicating possible intermediate competition. Higher titers of GAGs and higher supernatant to

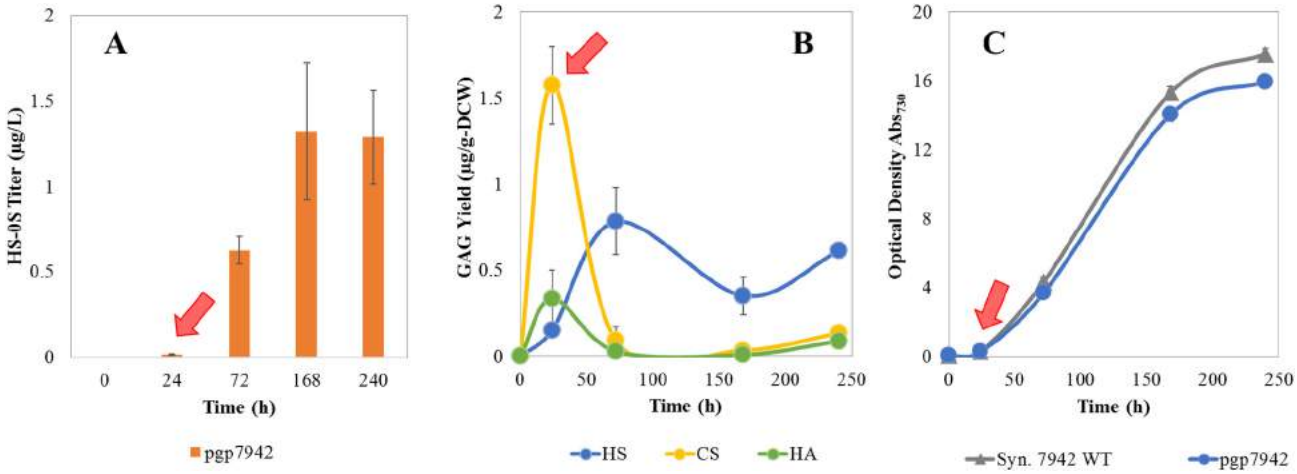


**Fig. 4.** Heparosan producing strain, *pgp7942*, under continuous 250 photons µmol/m$^2$/s (HL) and 0.2% (v/v) $CO_2$ (HC) shake flask conditions. Red arrow indicates time of induction with 1 mM IPTG. (A) Titer of heparosan (HS-0S) as measured in the lyophilized cell pellet. (B) Yield of glycosaminoglycans, heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronic acid (HA), produced in the pellet of strain, *pgp7942*. (C) Growth curve for the heparosan producing strain, *pgp7942*, and the wild type (WT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

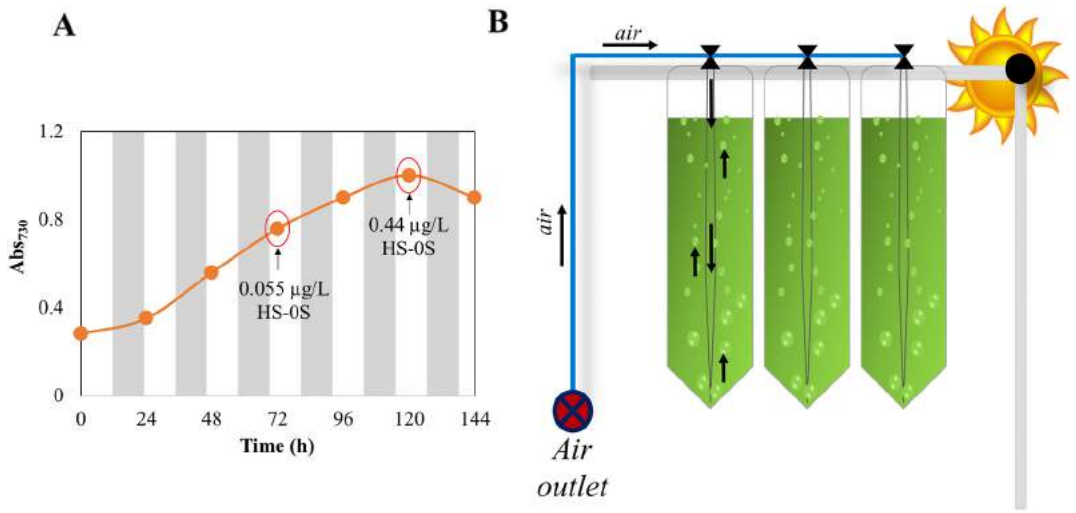


**Fig. 5.** Natural light cultivation of *Synechococcus* 7942 transformants. (A) Growth curve (as measured by absorbance at 730 nm) with the transformants *pgp7942* grown under natural diurnal light (max. 1200 ± 100 µmol/m$^2$/s), atmospheric $CO_2$ conditions (0.04% $CO_2$), and temperature 33 ± 10 °C. Heparosan titer (µg/L) by *pgp7942* at 72 h and 120 h is marked *via* red circles. (B) Bubble column plastic bag reactor set-up at Environmental laboratory. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pellet ratios were obtained in *pgp7942* cultures grown under continuous 250 µmol/m$^2$/s of light and 0.2% $CO_2$, most likely correlated to biomass increase and secretion of product under optimal growth conditions.

Compared to shake flask cultures of recombinant *B. subtilis* [10], the titer and yield reported here is much lower. HS-0S yields on biomass and carbon substrate from *pgp7942* have reached 0.72 ± 0.15 µg/g-DCW (0.83 µg/g-C, using an estimated $CO_2$ uptake of 1 mmol/g-DCW/h), while engineered *B. subtilis* achieved at 90 mg/g-C. This is not surprising since heterotrophic bacteria can grow into much higher cell density and use sugar instead of $CO_2$ to directly synthesize polysaccharides.

Moreover, WT and *pgp7942* cultures were analysed over time for 10 days under HL and HC conditions. Productivity (Table 2) and titer in *pgp7942* increased with dry cell weight, and following IPTG induction, HS yield was improved over CS and HA in *pgp7942*. HS-0S yield was highest during early exponential growth and levelled off after 72 h of growth at 0.72 ± 0.15 µg/g-DCW (Fig. 4). These time series cultures displayed significant improvement in HS-0S titer over WT (> 0.03 µg/L). Genetic modification nor heparosan accumulation negatively affected the growth of the HS-0S producing strain.

Following these experiments under controlled laboratory conditions, recombinants were grown under natural diurnal light (light 1200 ± 100 µmol/m$^2$/s, temperature 33 ± 10 °C) at a 5 L scale (Fig. 5). Natural light scale-up studies with *pgp7942* produced a maximum of 0.44 µg/L (0.5 µg/g-DCW) of HS-0S at 120 h with a productivity of 0.09 µg/L/day (Fig. 5). EL productions indicates effective functionality of recombinants under natural light and scaled conditions (Table 2). The cultivation experiments demonstrate similar titers under scaled-up conditions and thus the possible use of this strain for low-cost biomanufacturing of a high-value product. For struggling algal bio-refineries and farmers, this is significant for increasing profit margins and promoting green local economies.

This study observed that PCC 7942 can synthesize multiple GAGs (Fig. 3B) with WT naturally producing chondroitin sulfate (CS). Following induction of the genes *galU* and *PmHS2*, *pgp7942* produced more HS-0S than CS (Fig. 4B), indicating competition between polysaccharide synthesis pathways (Fig. 3C). Interestingly, PCC 7942 does not have an annotated gene for the conversion of UDP-*N*-

acetylglucosamine to UDP-*N*-acetylgalactosamine, one of the essential precursors for CS (Fig. 1), nor have an annotated gene for CS synthase. Since cyanobacteria are able to produce a wide range of GAG-like compounds, such as extracellular polymeric substances (EPS) and lipopolysaccharides (LPS), it is likely that there are unannotated genes and promiscuous enzymes responsible for complex carbohydrates. For example, *E. coli* K4 possess a CS synthase (*WP_000025667*) [34] and *BLASTp* showed 27% homology to PCC 7942's cellulase synthase. Further gene function studies are required to identify a CS pathway in PCC 7942.

## 4. Conclusion

PCC 7942 recombinants, *pgp7942*, were successfully developed for the photoautotrophic production of heparosan through homologous recombination of the genes *galU* and *PmHS2*. *pgp7942* cells were cultivated under different environmental conditions yielding maximum heparosan production (2.8 μg/L) with high light and high $CO_2$, which was $10^3$ folds higher than that in wild type strain. PCC 7942 release as well as store GAGs and hence, could serve as efficient biosynthetic platforms for continuous production of glycans like heparosan at a commercial scale. Our scaled culture under natural light conditions demonstrates this platform for the photosynthetic production of high-value chemicals from low-cost inputs. Further research efforts are needed for polymer characterization and regulation; however this study presents proof-in-concept for cyanobacteria as a platform for complex carbohydrate synthesis.

## Conflict of interest

The authors declare no competing financial interests.

## Statement of informed consent, human or animal rights

Not applicable.

## Author contributions

YJT, MK and RP initiated and designed the project. AS, MHA, BC and AL performed strain engineering, and AS and MHA carried out cyanobacteria cultivations. XH, YO, KX, YC, FZ and RJL performed product analysis. All authors agree to authorship and approve the final manuscript for submission.

## Acknowledgements

This project is supported by U.S. Department of Energy-BER (DESC0012722) and National Science Foundation CBET award number 1604547. Authors are also thankful for the support of international collaborations from McDonnell Academy Global Energy and Environment Partnership at Washington University.

## References

[1] A. Badri, A. Williams, R.J. Linhardt, M.A.G. Koffas, The road to animal-free glycosaminoglycan production: current efforts and bottlenecks, Curr. Opin. Biotechnol. 53 (2017) 85–92.
[2] A.A.E. Chavaroche, L.A.M. Van Den Broek, G. Eggink, Production methods for heparosan, a precursor of heparin and heparan sulfate, Carbohydr. Polym. 93 (1) (2013) 38–47.
[3] B.F. Cress, J.A. Englaender, W. He, D. Kasper, R.J. Linhardt, M.A.G. Koffas, Masquerading microbial pathogens: capsular polysaccharides mimic host-tissue molecules, FEMS Microbiol. Rev. 38 (4) (2014) 660–697.
[4] S. Peterson, A. Frick, J. Liu, Design of biologically active heparan sulfate and heparin using an enzyme-based approach, Nat. Prod. Rep. 26 (5) (2009) 610.
[5] A. Williams, R.J. Linhardt, M.A.G. Koffas, Metabolic engineering of capsular polysaccharides, Em. Top. Life Sci. 2 (3) (2018) 337–348.
[6] D. Vaidyanathan, A. Williams, J.S. Dordick, M.A.G. Koffas, R.J. Linhardt, Engineered heparins as new anticoagulant drugs, Bioeng Transl. Med. 2 (1) (2017) 17–30.
[7] J. Liu, R.J. Linhardt, Chemoenzymatic synthesis of heparan sulfate and heparin, Nat. Prod. Rep. 31 (12) (2014) 1676–1685.
[8] M. Suflita, L. Fu, W. He, M. Koffas, R.J. Linhardt, Heparin and related polysaccharides: synthesis using recombinant enzymes and metabolic engineering, Appl. Microbiol. Biotechnol. 99 (18) (2015) 7465–7479.
[9] Z. Wang, J.S. Dordick, R.J. Linhardt, *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. 2011, Bioeng. Bugs 2 (1) (2011) 1–5.
[10] P. Jin, L. Zhang, P. Yuan, Z. Kang, G. Du, J. Chen, Efficient biosynthesis of polysaccharides chondroitin and heparosan by metabolically engineered *Bacillus subtilis*, Carbohydr. Polym. 140 (2016) 424–432.
[11] R.S. Lane, F.M. Haller, A.A.E. Chavaroche, A. Almond, P.L. Deangelis, Heparosan coated liposomes for drug delivery, Glycobiology 27 (11) (2017) 1062–1074.
[12] J.A. Englaender, Y. Zhu, A.N. Shirke, L. Lin, X. Liu, F. Zhang, R.A. Gross, M.A. Koffas, R.J. Linhardt, Expression and secretion of glycosylated heparin biosynthetic enzymes using *Komagataella pastoris*, Appl. Microbiol. Biotechnol. 101 (7) (2017) 2843–2851.
[13] W. He, L. Fu, G. Li, J.A. Jones, R.J. Linhardt, M. Koffas, Production of chondroitin in metabolically engineered *E.coli*, Metab. Eng. 27 (2015) 92–100.
[14] W. Wang, J.A. Englaender, P. Xu, K.K. Mehta, J. Suwan, J.S. Dordick, F. Zhang, Q. Yuan, R.J. Linhardt, M. Koffas, Expression of low endotoxin 3-O-sulfotransferase in *Bacillus subtilis* and *Bacillus megaterium*, Appl. Biochem. Biotechnol. 171 (4) (2013) 954–962.
[15] B. Wu, N. Wei, V. Thon, M. Wei, Z. Yu, Y. Xu, X. Chen, J. Liu, P.G. Wang, T. Li, Facile chemoenzymatic synthesis of biotinylated heparosan hexasaccharide, Org. Biomol. Chem. 13 (18) (2015) 5098–5101.
[16] F. Zhang, B. Yang, M. Ly, K. Solakyildirim, Z. Xiao, Z. Wang, J.M. Beaudet, A.Y. Torelli, J.S. Dordick, R.J. Linhardt, Structural characterization of heparins from different commercial sources, Anal. Bioanal. Chem. 401 (9) (2011) 2793–2803.
[17] A.A.E. Chavaroche, L.A.M. Van Den Broek, C. Boeriu, G. Eggink, Synthesis of heparosan oligosaccharides by *Pasteurella multocida* PmHS2 single-action transferases, Appl. Microbiol. Biotechnol. 95 (5) (2012) 1199–1210.
[18] P.L. DeAngelis, C.L. White, Identification of a distinct, cryptic heparosan synthase from *Pasteurella multocida* Types A, D, and F, J. Bacteriol. 186 (24) (2004) 8529–8532.
[19] A.M. Varman, Y. Yu, L. You, Y.J. Tang, Photoautotrophic production of D-lactic acid in an engineered cyanobacterium, Microb. Cell Factories 12 (2013) 117.
[20] Y. Yu, L. You, D. Liu, W. Hollinshead, Y.J. Tang, F. Zhang, Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory, Mar. Drugs 11 (8) (2013) 2894–2916.
[21] X. Lu, A perspective: photosynthetic production of fatty acid-based biofuels in genetically engineered cyanobacteria, Biotechnol. Adv. 28 (6) (2010) 742–746.
[22] M.H. Abernathy, J. Yu, F. Ma, M. Liberton, J. Ungerer, W.D. Hollinshead, S. Gopalakrishnan, L. He, C.D. Maranas, H.B. Pakrasi, D.K. Allen, Y.J. Tang, Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis, Biotechnol. Biofuel. 10 (2017) 273.
[23] R. De Philippis, M. Vincenzini, Exocellular polysccharides from cyanobacteria and their possible applications, FEMS Microbiol. Rev. 22 (1998) 151–175.
[24] J.-C. Kehr, E. Dittmann, Biosynthesis and function of extracellular glycans in cyanobacteria, Life 5 (1) (2015) 164–180.
[25] S. Singh, B.N. Kate, U.C. Banecjee, Bioactive compounds from cyanobacteria and microalgae: an overview, Crit. Rev. Biotechnol. 25 (3) (2005) 73–95.
[26] A. Sarnaik, R. Pandit, A. Lali, Growth engineering of *Synechococcus elongatus* PCC 7942 for mixotrophy under natural light conditions for improved feedstock production, Biotechnol. Prog. 33 (5) (2017) 1182–1192.
[27] E.M. Clerico, J.L. Ditty, S.S. Golden, Specialized techniques for site-directed mutagenesis in cyanobacteria, Methods Mol. Biol. 362 (2007) 155–171.
[28] R.M.M. Abed, S. Dobretsov, K. Sudesh, Applications of cyanobacteria in biotechnology, J. Appl. Microbiol. 106 (1) (2009) 1–12.
[29] K. Maeda, R. Narikawa, M. Ikeuchi, CugP is a novel ubiquitous non-GalU-type bacterial UDP-glucose pyrophosphorylase found in cyanobacteria, J. Bacteriol. 196 (13) (2014) 2348–2354.
[30] T. Monshupanee, A. Incharoensakdi, Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium *Synechocystis* sp. PCC 6803, J. Appl. Microbiol. 116 (2013) 830–838.
[31] J. Espinosa, K. Forchhammer, A. Contreras, Role of the *Synechococcus* PCC 7942 nitrogen regulator protein PipX in NtcA-controlled processes, Microbiology 153 (3) (2007) 711–718.
[32] M. Ahmed, T.C.W. Moerdijk-Poortvliet, A. Wijnholds, L.J. Stal, S. Hasnain, Isolation, characterization and localization of extracellular polymeric substances from the cyanobacterium *Arthrospira platensis* strain MMG-9, Eur. J. Phycol. 49 (2) (2014) 143–150.
[33] S. Pereira, R. Mota, C.P. Vieira, J. Vieira, P. Tamagnini, Phylum-wide analysis of genes/proteins related to the last steps of assembly and export of extracellular polymeric substances in cyanobacteria, Sci. Rep. 5 (2015) 14835.
[34] T. Ninomiya, N. Sugiura, A. Tawada, K. Sugimoto, H. Watanabe, K. Kimata, Molecular cloning and characterization of chondroitin polyermase from *Escherichia coli* strain K4, J. Biol. Chem. 277 (2002) 21567–21575.