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

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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 pUPlm48 for cloning galU and PmlBS2 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 uridylytransferase 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.

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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 hyaluronic 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 co-polymer 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
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 kfc and kfiA genes from E. coli K5, yet molecular weight and homogeneity were difficult to control [10,16]. Nevertheless, Pasteurellula multocida possesses a PmHS2 gene that encodes for a dual functional heparosan synthase that replaces the two genes, kfc 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 their 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 TuKaRa 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 (AllianaBio, 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 (HMedia) [26]. For PCC 7942 cultivation, the seed cultures were grown with continuous illumination of 55 ± 2 μmol/m²/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) CO₂ (HC) at 37 °C and continuous light environments (100 photons μmol/m²/s (LL)) and 250 photons μmol/m²/s (HL)). Low nitrogen cultures were prepared with BG-11 medium with 1/5 NaN₃O (0.15 g/L) to promote carbohydrate accumulation, while 30 mM of glycerol was added to cultures for photoheterotrophic 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/m²/s, 33 ± 10 °C) in a bubble column plastic bag reactor (air bubbling rate of 60 mL/min) using atmospheric CO₂ at a 5 L culture volume. Culture pellet was analysed 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
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-GalNAc4S; ΔUA2S-GalNAc6S; ΔUA2S-GalNAc4S6S; ΔUA-GalNAc4S6S; ΔUA2S-GalNAc4S6S), unsaturated disaccharide standards of HS (ΔUA-GlcNAc; ΔUA-GlcrNS; ΔUA-GlcNAc6S; ΔUA-GlcNAc4S6S; ΔUA2S-GalNAc4S6S), 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).
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 Sartorius Stedim Biotech (Bohemia, NY, USA).

2.4.2. GAG extraction, sample desalination and digestion

Lyophilized pellets were re-suspended with 15 mL of ddH₂O, then sonicated for 10 min in an ice bath using a Misonix Sonicator 3000 (300 watts of energy deliver) with microtip at 40% magnitude, followed by sonication 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 extracts were re-dissolved in 400 μL of distilled water and de-salted by passing through a 3 kDa molecule weight cut-off filter unit. Recombinant heparin lyase I, II, III (pH optima 7.0 to pH 7.0) was added to the samples, pH optimum 7.4) were added to each sample and mixed well. The digestion was terminated by removing the enzymes by centrifugation. The supernatants were all placed at 37 °C for 12 h, after which enzymatic digestion was terminated by removing the enzymes by centrifugation.

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 EC18 (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 FKA (glucosaminyl transferase) and FKC (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 acid...
Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition description</th>
<th>Carbon source</th>
<th>Light</th>
<th>Heparosan Productivity (μg/L/day) (n = 2)</th>
<th>Biomass productivity (at mid-log phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgp7942</td>
<td>Shaking flask, HC/HL</td>
<td>0.2% CO₂ (atmospheric) + 30 mM glycerol</td>
<td>100 μmol/m²/s (continuous)</td>
<td>0.09 ± 0.01</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>WT</td>
<td>Shaking flask, HC/HL</td>
<td>0.2% CO₂ (atmospheric)</td>
<td>250 μmol/m²/s (continuous)</td>
<td>&lt; 0.01</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>pgp7942</td>
<td>Natural scale-up*</td>
<td>0.04% CO₂ (atmospheric)</td>
<td>Natural diurnal light</td>
<td>0.09</td>
<td></td>
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</tbody>
</table>

*Experiment carried out under natural diurnal conditions in environmental laboratory.

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 UDP-glucose-1-phosphate uridylyltransferase, which synthesizes UDP-glucose, same as *galU* [29]. Protein BLAST (BLASTp) of this non-*galU* type uridylyltransferase exhibited 99% sequence similarity with mannos-1-phosphate guanylyltransferase (BAM549031.1) from PCC 6803 (SI Fig. 1). Its *NTP_transferase* functional domain belonging to *Glyco-transfer*, *GTA_type* superfamily was verified from Conserved Domain database at NCBI (domain architecture ID 11440233). BLASTp of this domain displayed 79% sequence homology with mannos-1-phosphate guanylyltransferase (ABBSO0031.1, gene: SYNPC7942_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 mannos-1-phosphate guanylyltransferase (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 heparan production than native RS10005 alone.

Cyanobacteria tend to synthesize polysaccharides under various physical and chemical stress conditions [23]. Therefore, to improve heparan (HS-0S) production from *pgp7942*, cells were grown under different environmental conditions (Fig. 2C), such as high CO₂ (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* production (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 heparan, 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
pellet ratios were obtained in pgp7942 cultures grown under continuous 250 μmol/m²/s of light and 0.2% (v/v) CO₂, 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 CO₂ 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 CO₂ 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 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/m²/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 titer 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-
acetylgalactosamine 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 possesses a CS synthase (WP.0000255667) [34] and BLASTp showed 27% homology to PCC 7942's cellulose 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 heparan through homologous recombination of the genes *gali* and *PmHS2*. pgp7942 cells were cultivated under different environmental conditions yielding maximum heparan production (2.8 mg/L) with high light and high CO2, which was 103 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 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 cultivation. XY, YO, KY, YC, FZ and RJL performed product analysis. All authors agree to authorship and approve the final manuscript for submission.

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