Expression of enzymes for 3′-phosphoadenosine-5′-phosphosulfate (PAPS) biosynthesis and their preparation for PAPS synthesis and regeneration

Payel Datta1,2 · Li Fu1,2 · Wenqin He1,2 · M. A. G. Koffas1,2,3 · J. S. Dordick1,2,3,4 · R. J. Linhardt1,2,3,4

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Abstract
The synthesis of sulfated polysaccharides involves the sulfation of simpler polysaccharide substrates, through the action of sulfotransferases using the cofactor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Three enzymes are essential for the in vitro synthesis of PAPS, namely, pyrophosphatase (PPA), adenosine 5′-phosphosulfate kinase (APSK), and ATP sulfurylase (ATPS). The optimized enzyme expression ratio and effect on PAPS synthesis were evaluated using ePathBrick, a novel synthetic biology tool that assemble multiple genes in a single vector. The introduction of multiple promoters and stop codons at different location enable the bacterial system to fine tune expression level of the genes inserted. Recombinant vectors expressing PPA (U39393.1), ATPS (CP021243.1), and PPA (CP047127.1) were used for fermentations and resulted in volumetric yields of 400–1380 mg/L with accumulation of 34–66% in the soluble fraction. The enzymes from soluble fraction, without any further purification, were used for PAPS synthesis. The PAPS was used for the chemoenzymatic synthesis of a heparan sulfate polysaccharide and coupled with a PAPS-ASTIV regeneration system. ASTIV catalyzes the regeneration of PAPS. A recombinant vector expressing the enzyme ASTIV (from Rattus norvegicus) was used for fermentations and resulted in volumetric yield of 1153 mg/L enzyme with accumulation of 48% in the soluble fraction. In conclusion, we have successfully utilized a metabolic engineering approach to optimize the overall PAPS synthesis productivity. In addition, we have demonstrated that the ePathBrick system could be applied towards study and improvement of enzymatic synthesis conditions. In parallel, we have successfully demonstrated an autoinduction microbial fermentation towards the production of mammalian enzyme (ASTIV).

Key points
• ePathBrick used to optimize expression levels of enzymes.
• Protocols have been used for the production of recombinant enzymes.
• High cell density fed-batch fermentations with high yields of soluble enzymes.
• Robust fermentation protocol successfully transferred to contract manufacturing and research facilities.

Keywords Heparin · 3′-phosphoadenosine-5′-phosphosulfate (PAPS) · PAPS-ASTIV recycling system · Fed-batch fermentation · High cell density autoinduction · ePathBrick platform

Introduction
Sulfation of biomolecules is ubiquitous in eukaryotic cells and plays a pivotal role in various biological processes involving homeostasis and hormone metabolism; inactivation and drug metabolism; activation and inactivation of mutagens and xenobiotics; and post-translational modification of proteins, carbohydrates, and polysaccharides (Günel et al. 2019; Hale et al. 2020; Italia et al. 2020; Kamiyama et al. 2003; Klaassen and Boles 1997; Koprivova and Kopriva 2016; Lin 2004; Piecewicz and...
The sulfation of biomolecules is catalyzed by tissue-specific sulfotransferases found in the Golgi and cytosol of eukaryotes (Coughtrie 2016; Goettsch et al. 2006; Kamiyama et al. 2003). The cofactor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) is nearly universally used as a sulfate donor in the sulfation of a target biomolecule (Badri et al. 2019; Burkart et al. 2000; Zhou et al. 2011). Therefore, the synthesis and regeneration of PAPS are critical for the action of sulfotransferases and their use in biosynthesis. PAPS is synthesized in the cytosol, through the sequential action of adenosine-triphosphate (ATP) sulfurylase (ATPS, ATP sulfate adenylyltransferase) and adenosine-5′-phosphosulfate (APS) kinase (APSK, ATP adenylylsulfate 3′-phosphotransferase) (Burkart et al. 2000; Zhou et al. 2011). ATPS catalyzes the sulfation of ATP with inorganic sulfate to yield (APS) and inorganic pyrophosphate (PPi) (Fig. 1). The APS is then converted to PAPS by APSK in the presence of ATP (Burkart et al. 2000; Zhou et al. 2011). The resulting PAPS is then either utilized by cytosolic sulfotransferases or translocated into the Golgi (Schwarz et al. 1984) for use by Golgi-associated sulfotransferases (Coughtrie 2016; Goettsch et al. 2006; Kamiyama et al. 2003).

Understanding the biochemistry and activity of enzymes involved in PAPS synthesis is critical for myriad applications, including the cost-efficient chemoenzymatic synthesis of sulfated polysaccharides like HS and heparin (Badri et al. 2018; Zhang et al. 2009). HS is important in signaling in developmental biology, physiology, and pathophysiology (Lindahl et al. 1998; Soares da Costa et al. 2017; Zhang et al. 2009). Heparin is important pharmacologically as an anticoagulant drug (Linhardt 2003). The biosynthesis of HS/heparin biosynthesis takes place in the Golgi of mammalian mast cells and involves the chemical N-deacetylation, N-sulfation of heparosan to afford N-sulfoheparosan, followed by epimerization of the glucuronic acid residues to iduronic acid, catalyzed by C5-epimerase, and a series of HS-sulfotransferase-catalyzed reactions at the 2, 6, and 3-OH positions in the presence of PAPS (Liu and Linhardt 2014; Zhang et al. 2009).

An effective chemoenzymatic process requires the sufficient production of the three enzymes involved in PAPS synthesis as well as ASTIV involved in PAPS recycling. To this end, we have focused on enzyme production using high cell density fed-batch fermentations.

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**Fig. 1** Enzymes in PAPS synthesis and regeneration system. a PAPS is synthesized through the addition of inorganic sulfate to ATP and the addition of phosphate to APS (Burkart et al. 2000; Zhou et al. 2011). b Once generated, PAPS serves as a donor for sulfotransferases and can be regenerated in vitro using p-nitrophenol sulfate (PNPS) as a sacrificial sulfate donor.
In addition, we demonstrate that these enzymes can be used in the chemoenzymatic synthesis of defined HS polysaccharides.

**Materials and methods**

**Materials, bacterial strains, and plasmid constructs**

Chemicals for media formulation, enzyme purification, enzyme quantification, and PAPS production were purchased from Sigma-Aldrich (St. Louis, MO). Antibiotics and IPTG were purchased from Gold Biotechnology (St. Louis, MO). The bacterial strains used in the study are listed in Table 1.

**High cell density non-autoinduction fed-batch fermentation of PAPS synthesis enzyme**

Enzyme fermentations were performed in 5-L BioFlo 320 Eppendorf or 1-L Applikon bioreactors. The fermentations consisted of a batch phase, followed by a fed-batch phase. The batch media and feed compositions for each strain are shown in Table 2. The batch phase was initiated with the inoculation of seed culture in batch media (starting OD600 =

### Table 1  Strain, gene sequence accession numbers, and plasmid information (see Table S1 for primer information and cloning strategy for the genes ATPS, APSK and PPA)*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>General cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21star™ (DE3)</td>
<td><em>F.ompT hsdS30 (r6, mcr) galdcmrel131</em> (DE3)</td>
<td>Novagen</td>
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<tr>
<td>Rosetta-gami B (DE3)</td>
<td><em>F.ompT hsdS30 (r6, mcr) gal dcm lacY1 ahpC</em> (DE3) <em>gor522::Tn10 trxB</em> pRARE (Cam&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Novagen</td>
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</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>Accession numbers</th>
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<td>U39393.1</td>
</tr>
<tr>
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<td><em>Kluyveromyces lactis</em></td>
<td>CP021243.1</td>
</tr>
<tr>
<td>PPA</td>
<td><em>Kluyveromyces lactis</em></td>
<td>CP047127.1</td>
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<table>
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<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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</thead>
<tbody>
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<td>pETM6</td>
<td>T7 promoter, ColE1 ori, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Xu et al. 2012)</td>
</tr>
<tr>
<td>pETM6_ATP</td>
<td>pETM6 carrying gene atps</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_ATPSH</td>
<td>pETM6 carrying gene atps fused with His tag in N-terminus</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_APSK</td>
<td>pETM6 carrying gene apsk</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_APSKH</td>
<td>pETM6 carrying gene apsk fused with His tag in N-terminus</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PPA</td>
<td>pETM6 carrying gene ppa</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PPA</td>
<td>pETM6 carrying gene ppa fused with His tag in N-terminus</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PS KP</td>
<td>pETM6 carrying genes in the order of atps, apsk ppa in pseudo-operon configuration</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PSPK</td>
<td>pETM6 carrying genes in the order of atps, apsk and ppa in pseudo-operon configuration</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PKSP</td>
<td>pETM6 carrying genes in the order of apsk, atps, and ppa in pseudo-operon configuration</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PKPS</td>
<td>pETM6 carrying genes in the order of ppa and atps in pseudo-operon configuration</td>
<td>This study</td>
</tr>
<tr>
<td>pETM6_PPKS</td>
<td>pETM6 carrying genes in the order of ppa, apsk and atps in pseudo-operon configuration</td>
<td>This study</td>
</tr>
<tr>
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<td>pCDFDuet-1 carrying gene ppa</td>
<td>This study</td>
</tr>
<tr>
<td>pETDuet-1_ATPS_APS K</td>
<td>pETDuet-1 carrying genes ATPS and APSK</td>
<td>This study</td>
</tr>
<tr>
<td>pET28a(+)_ASTIV</td>
<td>pET28a(+) carrying gene ASTIV from <em>R. norvegicus</em></td>
<td>Gift from Pr. Jian Lu, UNC</td>
</tr>
<tr>
<td>pMalc2x_6OST3</td>
<td>pMalc2x carrying the catalytic domain of mouse 6OST-3 (Pro121–Pro450)</td>
<td>(Chen et al. 2005)</td>
</tr>
</tbody>
</table>
Quantitative SDS-PAGE analysis with bovine serum albumin

Enzyme concentrations were determined using semi-quantitative SDS-PAGE analysis with bovine serum albumin (BSA) used as a standard. Briefly, the cell pellet (5 g wet weight) was dispersed in 25 mL of purification buffer (25 mM Tris-HCl (Bio-Rad, USA), 500 mM NaCl (Sigma, USA), pH 7.5) containing 8000 kU/L DNAse I (Sigma, USA), and protease inhibitors (SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free, Sigma, USA). The cells were lysed using sonication, followed by centrifugation at 13,500 × g for 40 min at 4 °C. The cell lysate (soluble enzyme fraction) was carefully transferred to a clean tube, and the cell pellet (insoluble fraction) was dissolved in 2.5 mL lysis buffer. Both the undiluted and the diluted fractions were evaluated using SDS-PAGE performed using a MiniProtean Tetra system (Bio-Rad, Hercules, CA). Denatured enzymes (10 μL of a 1:1 (v:v) boiled enzyme solution and sample buffer) and protein ladder (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards, Bio-Rad) were resolved using a 4–15% precast gel (Mini protean TGX gels, Bio-Rad) at 220 V. Gels were washed with deionized water, stained with GelCode™ Blue Safe Protein Stain (ThermoFisher, City, State), and destained with deionized water.

**PAPS production using APSK, ATPS, and PPA**

PAPS synthesis was performed using a modified protocol, based on a previously described method (Burkart et al. 2000; Zhou et al. 2011). Briefly, following fermentation, cells were harvested by centrifugation at 5000 × g for 20 min at 4 °C. The supernatant was discarded, and the cell paste was stored in −80 °C. On the day of the experiment, PPA expressing cells and ATPS+APSK expressing cells were thawed in lysis buffer (20 mM tris(hydroxymethyl) amino (Tris)–HCl, 500 mM NaCl, pH 8.0) and lysed using a microfluidizer at 4 °C. After centrifugation at 12,000 × g for 20 min at 4 °C, the lysate was filtered using a 0.45-μm filter. The filtered cell lysate was utilized for PAPS synthesis. Briefly, the cell lysate (0.3 mg/mL PPA and 0.6 mg/ml APSK+ATPS) was added to

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### Table 2  Batch media and feed composition

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant E. coli BL21 Star™ (DE3) expressing ASTIV (kanamycin, 50 μg/ml) ASTIV</td>
<td>Autoinduction batch media</td>
<td>Ammonium chloride (31 mM), Glycerol (7 g/L), KH₂PO₄ (25 mM), K₂HPO₄ (25 mM), lactose (2 g/L), MgSO₄·7H₂O (1.8 g/L), sodium chloride (5 g/L), tryptone (10 g/L), yeast extract (12 g/L)</td>
</tr>
<tr>
<td>Recombinant E. coli BL21 Star™ (DE3) expressing PPA (streptomycin 50 μg/ml) and combinant E. coli BL21 Star™ (DE3) expressing ATPS and APSK (carbenicillin, 50 μg/ml)</td>
<td>Autoinduction feed</td>
<td>40% Glycerol, supplemented with 67 g/L NH₄Cl and 100 g/L lactose</td>
</tr>
<tr>
<td></td>
<td>Non-autoinduction batch media</td>
<td>Glycerol (7 g/L), MgSO₄·7H₂O (1.8 g/L), sodium chloride (5 g/L), tryptone (10 g/L), yeast extract (12 g/L)</td>
</tr>
<tr>
<td></td>
<td>Non-autoinduction feed</td>
<td>50% glycerol, supplemented with 67 g/L NH₄Cl and 100 g/L yeast extract</td>
</tr>
</tbody>
</table>

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0.25 ± 0.1. The fermentation and bioreactor conditions are provided in supporting information Table S2. The fed-batch phase was initiated when OD₆₀₀ reached 3.5 ± 2.0. The cells were fed at an initial feed rate of 2.5 mL/h/L and the feeding rate was adjusted based on fluctuations in pH and dissolved oxygen (DO) levels. The cells were induced at 22 °C and temperature was reduced to 22 °C. The cells were fed at an initial rate of 2.5 mL/h/L and the feeding rate was adjusted, based on fluctuations in pH and dissolved oxygen (DO) levels. The lactose in the feed served as the inducer and overnight induction at 22 °C was performed. Post-induction, the cells were harvested by centrifugation at 5000 × g for 20 min at 4 °C. The cell paste was weighed and stored at −80 °C.

**High cell density autoinduction fed-batch fermentation of ASTIV enzyme**

Enzyme fermentations were performed in 5-L BioFlo 320 Eppendorf or 1-L Applikon bioreactors. The fermentations consisted of a batch phase, followed by a fed-batch phase. The batch media and feed compositions for each strain are shown in Table 2. The batch phase was initiated with the inoculation of seed culture in batch media (starting OD₆₀₀ = 0.25 ± 0.1). The fermentation and bioreactor conditions are provided in supporting information Table S2. The fed-batch phase was initiated when OD₆₀₀ reached 3.5 ± 2.0 and temperature was reduced to 22 °C. The cells were fed at an initial rate of 2.5 mL/h/L and the feeding rate was adjusted, based on fluctuations in pH and dissolved oxygen (DO) levels. The lactose in the feed served as the inducer and overnight induction at 22 °C was performed. Post-induction, the cells were harvested by centrifugation at 5000 × g for 20 min at 4 °C. The cell paste was weighed and stored at −80 °C.

**Quantification of enzyme yield using SDS-PAGE analysis**

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a reaction mixture that contained variable concentrations of ATP sodium (0 mM to 22.5 mM), 100 mM Na₂SO₄, 10 mM MgCl₂, 10 mM LiCl, 50 mM Tris-HCl (pH 8.0), and 0.02% (w/v) sodium azide. The reaction was performed for 16 h at 30 °C. Following the reaction, the reaction mixture was centrifuged at 12,000×g for 20 min. The supernatant was removed, clarified, and subjected to ion exchange chromatography, wherein PAPS was eluted with 5 mM sodium phosphate, pH 8.0, and 500 mM NaCl. PAPS was desalted using size exclusion chromatography (Sephadex G-10 resin) using high-performance liquid chromatography (HPLC) grade water as the mobile phase. The desalted PAPS was lyophilized and concentrated in HPLC grade water, aliquoted, and stored at −80 °C. The final product was assessed for yield, PAPS purity, and concentration.

Analysis of PAPS by HPLC-MS

Liquid chromatography mass spectrometry (LC-MS) analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE). The instrument was equipped with a 6300 ion-trap and a binary pump and a Poroshell 120 EC-C18 column (2.0 × 100 mm, 2.7 μm, Agilent, USA) was used for the experiments. Eluent A contained water/acetonitrile (85:15, v/v) and eluent B contained water/acetonitrile (35:65, v/v). Both eluents were supplemented with 12 mM tributylamine (TrBA) and 38 mM NH₃. The reaction was performed for 16 h at 30 °C. Following the reaction, the reaction mixture was centrifuged at 12,000×g for 20 min. The supernatant was removed, clarified, and subjected to ion exchange chromatography (Sephadex G-10 resin) using high-performance liquid chromatography (HPLC) grade water as the mobile phase. The desalted PAPS was lyophilized and concentrated in HPLC grade water, aliquoted, and stored at −80 °C. The final product was assessed for yield, PAPS purity, and concentration.

ASTIV activity assay

The ASTIV activity assay was performed as previously described (Paul et al. 2012) with some modification using a Shimadzu UV-1650PC. Briefly, the enzyme was purified using Ni-NTA Sepharose and quantified using nanodrop spectroscopy at 280 nm. The activity was determined by incubating 0.1 mg of purified ASTIV with 5 mM PNPS, 0.05 mM 3'-adenosine-5'-phosphate (PAP), in phosphate-buffered saline (pH 7.0) (15 min, 37 °C, absorbance at 400 nm). The specific activity was calculated using an extinction coefficient of 1.05 × 10⁻² M⁻¹ cm⁻¹.

Production, purification, and immobilization of MBP-tagged-6OST-3 enzyme

Recombinant E. coli Rosetta-gami B (DE3) strain (Novagen, Cambridge, MA) expressing the plasmid pMalc2x_6OST3 (the catalytic domain of mouse 6OST-3, Pro121–Pro450) was used for production of MBP-tagged-6OST-3 (Chen et al. 2005). The non-autoinduction fermentation protocol for PAPS biosynthetic enzymes was used for production of the MBP-tagged-6OST3 enzymes. The cell pellet (5 g wet weight) of MBP-tagged-6OST-3 was dispersed in 25 mL of purification buffer (25 mM Tris-HCl (Bio-Rad, USA), 500 mM NaCl (Sigma), pH 7.5) containing 8000 kU/L DNAse I (Sigma), and protease inhibitors (SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free). The cells were lysed using sonication, followed by centrifugation at 13,500×g for 40 min at 4 °C. The enzymes from the soluble fraction were purified using 5 mL of amylose resin (NEB, USA) according to manufacturer’s instructions. The purity and MW of the enzyme was assessed using SDS-PAGE gel analysis.

6-O-sulfation of N-sulfo heparosan using ASTIV and PAPS recycling system

NSH was prepared using a method previously described (Wang et al. 2011). N-sulfoheparosan (NSH) (2 mg) was treated with 6OST-3, using a modified protocol, based on a previously described method (Xiong et al. 2013). The sulfation reaction was coupled with the PAPS recycling system that consisted of PNPS, PAPS, and ASTIV. The detailed reaction conditions were as follows: 0.1 mg/mL NSH, 0.5 mg/mL 6OST-3, and 10 mM and 250 μM of PNPS and PAPS, respectively. Reactions were incubated at 37 °C for 40 h in 50 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 7.2). After the reaction was complete, the mixture containing N-sulfos 6-O-sulfo heparosan (NS6S) was boiled for 10 min followed with 5000×g centrifugation (10 min) to spin down precipitated free enzymes. The supernatant was collected,
desalted, and freeze-dried for further analysis, including disaccharide analysis and NMR.

**Disaccharide compositional analysis**

NSH and NS6S samples (200 μg each) were completely digested using a mixture of heparinase I, II, and III (10 μL each) in 200 μL 50 mM ammonium acetate buffer (pH 7.2) at 37 °C for 12 h. The resulting disaccharides were recovered using 10 KDa molecular weight cut-off (MWCO) ultrafiltration units (Millipore, MA) and washed with deionized water. The ultrafiltrates containing disaccharides were collected and freeze-dried for disaccharide analysis.

Disaccharide analysis was carried out with high-pressure liquid chromatography (HPLC)–ultraviolet spectrometry (UV). The analyses were performed with a Shimadzu LC-20 AD pump, Shimadzu CBM-20A controller, Shimadzu SIL-20AHT auto-sampler, Shimadzu CTO-20 AC column oven, and a Shimadzu SPD-20AV UV detector (Shimadzu, Kyoto, Japan). A Spherisorb SAX chromatography column (4.0 × 250 mm, 5.0 μm, Waters) was equilibrated with 1.8 mM monobasic sodium phosphate (eluent A, pH 3.0) and followed with ingredient elution after injection using 1.8 mM monobasic sodium phosphate with sodium perchlorate (eluent B, pH 3.0). Disaccharide standards were purchased from Iduron (Manchester, UK). The weight/weight (w/w) percentage for each disaccharide was calculated. Peak areas were used for quantification.

**NMR spectroscopy**

NSH and NS6S samples (each 1.5 mg) were analyzed with 1D $^1$H nuclear magnetic resonance (NMR). The 1D 1H-NMR experiments were performed on a Bruker Advance II 600 MHz spectrometer (Bruker Bio Spin, Billerica, MA) with Topspin 2.1.6 software (Bruker). Samples were each dissolved in 0.5 mL D$_2$O (99.996%, Sigma-Aldrich) and freeze-dried repeatedly to remove the exchangeable protons. The samples were dissolved in 0.4 mL D$_2$O and transferred to NMR microtubes (outside diameter, 5 mm, Norell (Norell, Landisville, NJ)). All NMR experiments were carried out with the conditions as previously reported. (Fu et al. 2014; Fu et al. 2016)

**Results**

**Expression of PAPS synthesis enzymes and PAPS ePathbrick screening platform**

ATPS, APSK, and PPA are used for the *in vitro* synthesis of PAPS. The enzymes were cloned into the pETM6 vector, expressed as His-tagged enzymes in recombinant *E. coli* cells. All three enzymes, ATPS (57.3 KDa), APSK (24.6 KDa), and PPA (20.5 KDa) were expressed as His-tagged proteins in *E. coli* (Fig. 2).

PAPS expression analysis was evaluated using ePathBrick platform (He 2017; Xu et al. 2012). The ePathBricks is a versatile platform used for rapid design and optimization of metabolic pathways in *E. coli*; in the current study, the ePathbrick platform has been successfully used as a tool to study gene expression of PAPS synthesis enzymes. The PAPS ePathbrick platform demonstrated that the ratio of PPA, APSK, and ATPS impact PAPS production. Increasing ATPS concentration negatively impacted PAPS production (Fig. 3). Two approaches were evaluated: (1) three genes in a single plasmid pETM6_PSKP used for expression and (2) three genes placed in a pseudo-operon configuration in different gene orders to optimize PAPS production (He 2017). This expression system demonstrated the feasibility of crude
cell lysate in successful in vitro PAPS production (Figure S2) and the need to balance the expression of the three enzymes involved in vitro PAPS synthesis. The PAPS ePathbrick system was used to evaluate the impact of the expression levels of these three enzymes on PAPS production. Optimal PAPS production required the three genes (PPA, APSK, ATPS) to be placed in a pseudo-operon configuration (He 2017). The reactions were carried out without supplementing ATP after cell lysis. The optimal strains contained plasmid pETM6_PSPK, where APSK was most highly expressed, followed by PPA, and then ATPS (Fig. 3b). An optimal strain should be able to covert most of the ATP to PAPS. Based on these results, two recombinant E. coli strains were engineered; namely, (1) E. coli BL21 Star™ (DE3) strain expressing PPA and (2) E. coli BL21 Star™ (DE3) strain expressing APSK and ATPS. These two strains were used for scale-up fed-batch fermentation experiments.

**High cell density non-autoinduction fed-batch fermentation of PAPS synthesis enzyme**

High cell density, fed-batch fermentation of PAPS synthesis enzymes without autoinduction may be broadly classified into three growth phases: (1) seed inoculation and cell growth in batch media; (2) initiation of feed and growth of cells at the exponential growth rate; and (3) induction with IPTG. Two strains expressing the three PAPS synthesis enzymes were expressed using the same fermentation protocol. The fermentation profiles for the two strains are shown in Figure S3a (E. coli BL21 Star™ (DE3) strain expressing PPA), and Figure S4a (E. coli BL21 Star™ (DE3) strain expressing APSK and ATPS). The yield and MW of the enzymes from soluble fraction were analyzed and quantified. Enzymes were present in both soluble and insoluble fractions (Figures S3b and S4b). The high cell density fed-batch fermentation of

### Table 3  Fermentation and enzyme yield (see Figure S1 for schematic diagram and experimental plan; see Table S3 for calculations; # semi-quantitative SDS-PAGE analysis)

<table>
<thead>
<tr>
<th>Strains Fermentation strategy</th>
<th>ASTIV Fed-batch lactose induction</th>
<th>PPA IPTG induction (0.5 mM)</th>
<th>ATPS and APSK IPTG induction (0.5 mM)</th>
</tr>
</thead>
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<td>Total fermentation time</td>
<td>23 to 27 h</td>
<td>26 h</td>
<td>24 h</td>
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<tr>
<td>Total induction time</td>
<td>19 h</td>
<td>21 h</td>
<td>19 h</td>
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</tr>
<tr>
<td>Soluble enzyme yield &lt;sup&gt;a&lt;/sup&gt; (mg/g cell paste)</td>
<td>5.75</td>
<td>14.45</td>
<td>ATPS: 4.65</td>
</tr>
<tr>
<td>Volumetric enzyme yield in soluble fraction (mg/L fermentation broth)</td>
<td>555</td>
<td>908</td>
<td>ATPS: 199</td>
</tr>
<tr>
<td>Volumetric total enzyme yield in soluble and insoluble fraction (mg/L fermentation broth)</td>
<td>1153</td>
<td>1383</td>
<td>ATPS: 579</td>
</tr>
<tr>
<td>% Enzyme in soluble fraction</td>
<td>48 %</td>
<td>66 %</td>
<td>ATPS: 34%</td>
</tr>
<tr>
<td>Enzyme purification and utilization method</td>
<td>Ni-NTA resin purification</td>
<td>Soluble fraction</td>
<td>Soluble fraction</td>
</tr>
</tbody>
</table>
strain expressing PPA resulted in a volumetric yield of 1380 mg/L of fermentation broth (Table 3); and 34% of the PPA enzymes accumulated in the insoluble fraction (misfolded proteins or inclusion bodies) and the volumetric yield of PPA in the soluble fraction (correctly folded active proteins) was 910 mg/L (Table 3). The high cell density fed-batch fermentation of strain expressing both APSK and ATPS varied in volumetric yield and the percentage of enzyme in the soluble fractions (Figure S4b and Table 3). The high cell density fed-batch fermentation of APSK resulted in a volumetric yield of 420 mg/L of fermentation broth (Table 3) and 34% of the APSK enzymes accumulated in the insoluble fraction and the volumetric yield of APSK in the soluble fraction was 280 mg/L (Table 3). The high cell density fed-batch fermentation of ATPS resulted in a volumetric yield of 580 mg/L of fermentation broth (Table 3) and 66% of the ATPS enzymes accumulated in the insoluble fraction and the volumetric yield of ATPS in the soluble fraction was 200 mg/L (Table 3). Current PAPS synthesis involves the quantification of the PAPS enzymes from the soluble fraction and directly adding the filtered soluble fractions (that contain the PAPS synthesis enzymes) into the PAPS synthesis reaction mixture.

High cell density fed-batch autoinduction fermentation of ASTIV

High cell density fed-batch autoinduction fermentation of ASTIV can be broadly classified into two growth phases: (1) seed inoculation and cell growth in a batch media and (2) initiation of feed and growth of cells at exponential growth rate (Fig. 4a). The yield and MW of the enzymes from soluble fraction were analyzed and quantified. Results demonstrate the presence of ASTIV in both soluble and insoluble fractions (Fig. 4b). The high cell density autoinduction fed-batch fermentation resulted in a volumetric ASTIV yield of 1150 mg/L of fermentation broth (Table 3). However, approximately 52% of the enzyme accumulated in the insoluble fraction (inactive enzyme) and the volumetric yield of ASTIV in the soluble fraction (active enzyme) was 560 mg/L and most of the soluble ASTIV was immobilized using Ni-NTA resin (Figs. 4b, 5, and Figure S5).

Utilization of PAPS towards generation of defined oligosaccharides, using HS-6OST3 sulfotransferase and ASTIV regeneration system

PAPS produced using the PAPS synthesis enzymes can be successfully used in the synthesis of defined HS polysaccharides in the presence of a ASTIV-coupled PAPS regeneration system. To this end, the ASTIV was prepared as a His-tagged fusion protein. The HS biosynthetic enzyme, 6OST-3 was
prepared as a maltose binding protein (MBP) fusion. These proteins were immobilized on Ni-NTA Sepharose and amylose resins, respectively. NSH was treated with immobilized 6OST3 overnight at 37 °C. The sulfation reaction was coupled with PAPS recycling system consisting of (1) ASTIV, (2) PNPS as a sacrificial sulfo-donor, and (3) PAPS. After the reaction was complete, the reaction mixture was filtered to remove the beads and desalted using 1 KDa MWCO centrifugal ultrafiltration. The purified mixture was lyophilized for further disaccharide analysis and NMR analysis. The disaccharide compositions of NSH and NSH treated with 6OST3 are shown in Table 4. After treatment with 6OST3, in presence of PAPS recycling system, NSH affords 6-O-sulfated, N-sulfoheparosan (NS6S). The formation of NS6S was verified using 1D 1H-NMR (Fig. 6).

Discussion

The scalable production of enzymes using high cell density fermentation generally focuses on the cost-effective production of active biocatalysts. Upstream fermentation parameters, such as media design, can impact downstream purification and utilization of the enzymes. The current work demonstrates two different enzyme fermentation and utilization strategies:

Table 4 Disaccharide compositional analysis of NSH and NS6S

<table>
<thead>
<tr>
<th></th>
<th>0S</th>
<th>NS</th>
<th>6S</th>
<th>2S</th>
<th>NS6S</th>
<th>NS2S</th>
<th>2S6S</th>
<th>TriS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSH</td>
<td>0.8</td>
<td>99.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NS6S</td>
<td>0.7</td>
<td>13.1</td>
<td>0.0</td>
<td>0.0</td>
<td>86.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
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</table>

(1) fed-batch fermentation of PAPS biosynthetic enzymes without autoinduction, followed by utilization of the enzymes present in the soluble fraction; and (2) autoinduction fed-batch fermentation of ASTIV, followed by utilization of purified enzymes in a PAPS regeneration system. In both strategies, the first step requires minimizing feed and reagents in the fermentation. For example, trace metals were omitted and the batch medium was prepared using tryptone, yeast extract, and glycerol. The batch medium for autoinduction was designed to use ammonium chloride, sodium chloride, phosphate buffer, and lactose. Minimizing the number of ingredients ensures better fermentation control, reduces errors related to media formulation, scale-up, and technology transfer, and reduces the costs and inventory management. Fed-batch fermentation without autoinduction for the production of the PAPS synthesis enzymes, PPA, APSK and ATPS, relies on standard IPTG induction. This fermentation strategy is robust and has been widely used towards the production of many other enzymes with minor modifications for IPTG concentration, duration of induction, and induction temperature. A fed-batch fermentation strategy using lactose autoinduction for the production of ASTIV was inspired based on the shake flask experiments. Protein expression in shake flasks using complex or defined media supplemented with phosphate buffer, glucose, glycerol, lactose, and trace metals has been reported (Studier 2005). The fed-batch fermentation strategy for ASTIV utilized this strategy with modifications including the elimination of glucose and trace metals and an optimized ratio of tryptone and yeast extract.

Enzyme fermentations are best evaluated using the volumetric yield of active enzymes required to generate the desired product. The current study utilizes two approaches of enzyme utilization: (1) purification of enzymes using specific resin and using immobilized enzymes in the reaction of ASTIV-coupled PAPS regeneration system and (2) directly adding the soluble fraction to the reaction mixture in PAPS synthesis. The current HS polysaccharide synthesis involves purification of ASTIV from the soluble fraction using a Ni-NTA Sepharose resin, and the immobilized enzymes utilized for the ASTIV-coupled PAPS regeneration system. The high cell density autoinduction fed-batch fermentation of the strain expressing ASTIV resulted in a volumetric yield of 560 mg/L of fermentation broth, which comprises 48% of the total enzymes expressed in the soluble and insoluble fraction (Table 3). The current approach for PAPS synthesis involves quantification of the PAPS enzymes from the soluble fraction and directly adding filtered soluble fractions containing PAPS synthesis enzymes in the PAPS synthesis reaction mixture. After the fermentation to produce PAPS biosynthetic enzymes, the enzyme yield was calculated using a semi-quantitative SDS-PAGE (Table 3). The PAPS biosynthetic enzymes were expressed in two strains, E. coli BL21 Star™ (DE3) strain expressing PPA and E. coli BL21 Star™ (DE3) strain,

Fig. 5 Purified ASTIV on Ni-NTA Sepharose resin. Lane 1, protein ladder; lane 2, 250 μg/mL BSA standard; lane 3, 125 μg/mL BSA standard; lane 4, 62.5 μg/mL BSA standard; lane 5, purified ASTIV (37 KDa) enzyme.
expressing APSK and ATPS. The high cell density fed-batch fermentation of the strain expressing PPA resulted in a volumetric yield of 910 mg/L of fermentation broth, which comprises 66% of the total enzymes expressed in the soluble and insoluble fraction (Table 3). Fermentation of the strain co-expressing APSK and ATPS demonstrated variable expression of APSK and ATPS. The high cell density fed-batch fermentation for APSK resulted in a volumetric yield of 280 mg/L of fermentation broth with 66% of the total enzymes expressed in the soluble (active) and insoluble (inactive) fraction (Table 3). The high cell density fed-batch fermentation of ATPS resulted in a volumetric yield of 200 mg/L of fermentation broth, which comprises 34% of the total enzymes expressed in the soluble and insoluble fraction (Table 3). The expression of APSK and ATPS are desirable features because, preliminary experiments showed that decreased ATPS expression increased APSK expression favor PAPS production (Fig. 3).

We hypothesize that enzymes in the insoluble fraction consist primarily of unfolded, partially processed, or inactive enzymes and that these enzymes may be scavenged with additional purification steps, including enzyme refolding. There may be various factors that impact the accumulation of enzymes in the insoluble fraction. In analyzing PAPS biosynthetic enzymes and their fermentations, we speculate that smaller enzymes can easily fold and accumulate in the soluble (active) fraction (Palmer and Wingfield 2012). Both APSK (25 KDa) and ATPS (57 KDa) are produced in the same fermentation. However, there are differences in the volumetric yield of APSK (66% soluble) and ATPS (34% soluble). Similarly, PPA is smaller (21 KDa) and 66% of the enzyme is in the soluble fraction. The production of enzymes is often not straightforward and many factors can impact the production of the volumetric yield of active enzymes, including (1) expression of chaperones, (2) post-translational modifications, and (3) strain metabolism (Belval et al. 2015; Brylski et al. 2019; Ellis 2013; Jhamb and Sahoo 2012; Thomas et al. 1997). In this context, we have observed with other enzymes that under similar fermentation conditions, and either induction at 30 °C for 4 h or higher cell growth rate during induction, the majority of enzymes expressed accumulate in the insoluble fraction. Additionally, the carbon and nitrogen molar ratio during fermentation can impact enzyme production. Future research is geared towards evaluating strategies towards improving soluble enzymes utilization, including strain engineering, fermentation optimization, and purification of enzymes from insoluble fractions.

In conclusion, the paper demonstrated that the ePathbrick platform that had previously been used for metabolic engineering, could be successfully utilized to study in vitro enzymatic synthesis conditions. This application of ePathbrick platform could be applied for one-pot synthesis of polysaccharides, such as...
as heparin and heparan sulfate. Heparin and heparan sulfate are sulfated polysaccharides with tissue-specific sulfation domains. Tissue-specific sulfotransferases (and their isoforms) dictate the sulfation patterns on heparin and heparan sulfate. The ePathbrick tool could assemble seven-gene pathways (Xu et al. 2012) and could be utilized to evaluate effect of sulfotransferase expression ratio on sulfation patterns on heparin and heparan sulfate. In parallel, we have successfully demonstrated a fed-batch fermentation protocol (IPTG induction or non-autoinduction protocol) that could be utilized for expression of various enzymes, including sulfotransferases (e.g., 6OST-3). The protocol has been successfully utilized for fermentation, scale-up (100-L) and technology transfer (to industrial partners) of heparin biosynthetic enzymes (e.g., 2OST, C5 epimerase, 6-OST-1, 6OST-3, and 3OST-1). The media and feed composition are similar for all these fermentations, with variations in induction parameters (e.g., IPTG concentration, induction temperature, and induction time). Adapting all the biosynthetic enzymes in similar fermentation protocol enables us to control inventory management and cost. In addition, the paper successfully demonstrates an autoinduction microbial fed-batch fermentation strategy towards the production of the enzyme ASTIV. The successful fermentation strategies and reduction in cost of the production of enzymes (related to upstream) has enabled the successful pilot scale production and utilization of the enzymes.

**Authors’ contributions** RJL and JSD provided the funding for this study. RJL and PD planned the study. PD, RJL, and JSD wrote the manuscript. LF performed the fermentations. WH and MAGK designed and prepared the recombinant organisms. LF was responsible for the studies trial partners) of heparin biosynthetic enzymes (e.g., 2OST, C5 epimerase, 6-OST-1, 6OST-3, and 3OST-1). The media and feed composition are similar for all these fermentations, with variations in induction parameters (e.g., IPTG concentration, induction temperature, and induction time). Adapting all the biosynthetic enzymes in similar fermentation protocol enables us to control inventory management and cost. In addition, the paper successfully demonstrates an autoinduction microbial fed-batch fermentation strategy towards the production of the enzyme ASTIV. The successful fermentation strategies and reduction in cost of the production of enzymes (related to upstream) has enabled the successful pilot scale production and utilization of the enzymes.

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**Compliance with ethical standards** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

**References**


Coughtrie MWH (2016) Function and organization of the human cyto- 
solic sulfotransferase (SULT) family. Chem Biol Interact 259:2–7. https://doi.org/10.1016/j.chembiol.2016.05.005


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**Affiliations**

**Payel Datta**$^{1,2}$ · **Li Fu**$^{1,2}$ · **Wenqin He**$^{1,2}$ · **M. A. G. Koffas**$^{1,2,3}$ · **J. S. Dordick**$^{1,2,3,4}$ · **R. J. Linhardt**$^{1,2,3,4}$

$^1$ Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

$^2$ Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

$^3$ Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

$^4$ Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA