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Expression and secretion of glycosylated heparin biosynthetic enzymes using *Komagataella pastoris*

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Abstract Heparin, an anticoagulant drug, is biosynthesized in selected animal cells. The heparin biosynthetic enzymes mainly consist of sulfotransferases and all are integral transmembrane glycoproteins. These enzymes are generally produced in engineered *Escherichia coli* as without their transmembrane domains as non-glycosylated fusion proteins. In this study, we used the yeast, *Komagataella pastoris*, to prepare four sulfotransferases involved in heparin biosynthesis as glycoproteins. While the yields of these yeast-expressed enzymes were considerably lower than *E. coli*-expressed enzymes, these enzymes were secreted into the fermentation media simplifying their purification and were endotoxin free. The activities of these sulfotransferases, expressed as glycoproteins in yeast, were compared to the bacterially expressed proteins. The yeast-expressed sulfotransferase glycoproteins showed improved kinetic properties than the bacterially expressed proteins.

Keywords Heparin · Biosynthetic enzymes · Yeast expression · Bacterial expression · Kinetics

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Introduction

Heparin is the most commonly used clinical anticoagulant with annual sales that surpass $3 billion corresponding to over 100 tons annual production (Bhaskar et al. 2012; Cress et al. 2014). Heparin is commonly purified from porcine or bovine mucosal tissues. This puts a strain on livestock, especially in China where most heparin is produced (Mintz and Liu 2013; Suflita et al. 2015). In 2008, there was a contamination in a supply of heparin that resulted in over 100 deaths in the USA. This crisis suggested the value of producing a non-animal-sourced heparin (Bhaskar et al. 2012; Bhaskar et al. 2015).

Heparin is a glycosaminoglycan (GAG) comprised of variably sulfated 1,4-linked repeating disaccharide units of glucosamine acid (GlcN) and uronic acid that is biosynthesized in the mast cells of mammals (Lindahl et al. 1986). All of the enzymes required for the chemoenzymatic synthesis of heparin from the capsular polysaccharide heparosan can be expressed in *Escherichia coli* (Bhaskar et al. 2015). Heparosan, a precursor for heparin biosynthesis, can be purified from *E. coli* K5 or *Pasteurella multocida* and then chemically *N*-de-acetylated and *N*-sulfonated to afford *N*-sulfoheparosan (NSH) (DeAngelis et al. 2002; Wang et al. 2010; Cress et al. 2013). Next, C_5-epimerase can be used to epimerize the GlcA residue of heparosan to the IdoA residue predominantly found in heparin, and then the C2 position in IdoA is sulfated by 2-OST. A mixture of 6-OST-1 and 6-OST-3 then acts on the GAG to further modify the chains. Finally, 3-OST-1 sulfates the C3 position, resulting in the 3-*O*-sulfo pentasaccharide that is responsible for the anticoagulant activity of heparin.

There are multiple problems with expressing these proteins in the Gram-negative bacterium *E. coli*. First, *E. coli* produces

lipopolysaccharide endotoxins that elicit shock when introduced into the human bloodstream (Opal et al. 1999). There are chemical and physical methods for eliminating endotoxins, but this adds extra steps to the production process (Petsch 2000; de Oliveira Magalhães et al. 2007). A second limitation is difficulties with enzyme expression in *E. coli* as this organism is not well suited for protein secretion. Secretion of overexpressed proteins simplifies purification. Furthermore, there have been challenges associated with obtaining heparin biosynthetic enzymes, expressed in *E. coli*, with high activity and stability. This is probably due, at least in part, to the fact that all of the enzymes originate in mammals (Chen 2012).

One of the heparin biosynthetic proteins, 3-OST-1, was successfully expressed in the Gram-positive bacteria *Bacillus subtilis* and *Bacillus megaterium* in an effort to begin to address these problems (Wang et al. 2013). Both of these organisms have also been noted for their ability to secrete heterologous proteins in high quantities (Jiang et al. 2006; Yang et al. 2007; Biedendieck et al. 2007; Chen et al. 2010). Unfortunately, 3-OST-1 was not secreted from *Bacillus* and showed problems folding.

Sulfotransferases, involved in heparin biosynthesis, have been expressed and secreted from the yeast *Kluveromyces lactis* (Zhou et al. 2011). However, the researchers were unable to express active C_5-epimerase required for the biosynthesis of heparin. The enzymes generally had higher sulfotransferase activity than their *E. coli*-expressed enzyme counterparts and all but one of the enzymes had increased thermal stability. The obvious benefit to using a yeast expression system is that yeast are eukaryotes and able to post-translationally modify their proteins. Studies have shown that protein glycosylation can impact protein stability, activity, and expression levels (Zhou et al. 2009; Beckham et al. 2012; Kazenwadel et al. 2013; Zou et al. 2013; Han et al. 2014; Shirke et al., 2016a, b).

Komagataella pastoris, formerly *Pichia pastoris*, is a methylotrophic organism that has widely been used to express and secrete heterologous proteins. *K. pastoris* has also been recently used in the production of pharmaceutical proteins (Martínez et al. 2012). Heterologous proteins have been secreted in large amounts as high as 1 g/L in *K. pastoris* (Yang et al. 2013; Pyati et al. 2014; Shirke et al. 2016a). Furthermore, there are many commercial tools available for heterologous protein expression in *K. pastoris*, allowing for expression under different promoters as well as strains with altered protein glycosylation pathways. Here, we express and secrete all four of the sulfotransferases required for the chemoenzymatic synthesis of non-animal source heparin from *K. pastoris* and compare their activities to those of the *E. coli*-expressed enzymes.

Materials and methods

Strains and plasmids

Cloning and molecular biology was carried out in *E. coli* DH5α. Expression was performed in *K. pastoris* (ATCC 76273). Genes were cloned into *K. pastoris* suicide vector pJ912-19 (DNA 2.0). The heparin biosynthetic genes 3-OST-1, 2-OST, 6-OST-1, and, 6-OST-3 were cloned out of plasmids that were previously constructed for expression in *E. coli* (Xu et al. 2011; Bhaskar et al. 2015).

Construction of integration vectors

Four heparin biosynthetic genes were PCR amplified using primers 1–12 (Integrated DNA Technologies) (Table 1) and cloned into pJ912-19 at the *Xho*I and *Not*I restriction sites using normal cloning protocols. Cloning here results in the fusion of the genes with the alpha-mating factor, which targets the proteins for secretion from the cell. Each gene was cloned with a 6× His tag on the N-terminus to facilitate purification. In addition, each primer contained a sequence encoding the addition of a KR residue sequence before the protein sequence to facilitate cleavage of the alpha-mating factor. Cloning was verified by restriction digestion and Sanger Sequencing (Genewiz, Inc.).

Additionally, pJ912-19 was modified to allow cloning of the heparin biosynthetic genes. Most of these genes harbor an internal *Sac*I restriction site, which is the restriction enzyme that is normally used to linearize the DNA for chromosomal integration. Site-directed mutagenesis was performed using a standard protocol using primers 13 and 14 (Table 1) to change the *Sac*I site of pJ912-19 to a *Spe*I site, creating pJ912*. This mutagenesis was verified with Sanger sequencing (Genewiz, Inc.) The gene encoding 3-OST-1 was cloned into pJ912-19, while the remaining genes were cloned into pJ912*.

Growth media and buffer solutions

Growth of *E. coli* used for cloning was performed in Luria Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO) supplemented with appropriate antibiotics. For competent cell production and general growth, *K. pastoris* strains were grown in YPD broth (1% yeast extract, 2% peptone, 2% dextrose) and grown on YPDS plates (YPD with 2% agar and 1 M sorbitol) supplemented with appropriate antibiotics. Seed cultures for fermentations were grown in BMGY (1% yeast extract, 2% peptone, 10 mM potassium phosphate buffer pH 6.0, 0.34% yeast nitrogen base, 1% $(NH_4)_2SO_4$, 0.4 mg/mL biotin, 1% glycerol). Fermentations were carried out in basal salt media (BSM) containing 0.4% glycerol, 0.9 g/L $CaSO_4$, 14.67 g/L K_2SO_4, 11.67 g/L $MgSO_4{\cdot}7H_2O$, and 9 g/L $(NH_4)_2SO_4$. The trace salt solution used for feed solutions consisted of 6 g/L

Table 1 DNA primers used in this study

Number	Primer ID	Primer sequence (5′– > 3′)
1	3ostFOR	GGCGActcgagAAAAGGCATCATCATCATCATCATGGCACAGC
2	3ostREV	CGCCCgcggccgcCTATCAGTGCCAGTCGAATG
3	MBP2ostFOR	GGCGActcgagAAAAGGATGAAAATCGAAGAAGGTAAACTGGT AATCTGG
4	MBP2ostREV	CGCCCgcggccgcTCAGTTCGACTTCGGG
5	HIS2ostFOR	GGCGActcgagAAAAGGCACCATCATCACCACCACCGGGAAAT TGAACAGCGG
6	HIS2ostREV	CGCCCgcggccgcTCAGTTCGACTTCGGG
7	6ost1FOR	GGCGActcgagAAAAGGCACCATCATCACCACCACCATTACGA GAAAAAGTACTACTTCC
8	6ost1REV	CGCCCgcggccgcCTACTACCACTTCTCAATGATATGG
9	f6ost3FOR	GGCGActcgagAAAAGGCACCATCATCACCACCACATGCCGTC CTGTACCTC
10	f6ost3REV	CGCCCgcggccgcTTAGGGCCACCGGTGT
11	t6ost3FOR	GGCGActcgagAAAAGGCACCATCATCACCACCACCCCCGGTT CGTGCCTCGATTCAA
12	t6os3tREV	CGCCCgcggccgcTTAGGGCCACCGGTGT
13	pJ912sac-speFOR	AACCAGCCCAGTTATTGGGCTTGATTGactagtGCTCATTCCAATTC CTTCTATTAGGCT
14	pJ912sac-speREV	AGCCTAATAGAAGGAATTGGAATGAGCactagtCAATCAAGCCCAAT AACTGGGCTGGTT

$CuSO_4 \cdot 5H_2O$, 0.08 g/L NaI, 3 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 20.0 g/L $ZnCl_2$, 65 g/L $FeSO_4 \cdot 7H_2O$, and 0.2 g/L biotin. Glycerol feed for fermentations consisted of 50% (*v*/*v*) glycerol supplemented with 12 mL/L trace salt solution. Methanol feed consisted of 100% methanol supplemented with 12 mL/L trace salt solution. Control of pH was achieved with 28–30% NH_4OH (Fisher Scientific). For protein purification, 50 mM potassium phosphate pH 7.0 was supplemented with 5 or 100 mM imidazole for the wash and elution buffers, respectively. Digestion buffer for oligosaccharide analysis was 50 mM ammonium acetate and 2 mM $CaCl_2$ pH 7.0. Recombinant heparin lyase I, II, and III were expressed and purified from *E. coli* as previously described (Lohse and Linhardt 1992; Huang et al. 2012).

Transformation into *K. pastoris* and verification using colony PCR

Electro-competent *K. pastoris* cells were created following a standard protocol (Lin-Cereghino et al. 2005). Briefly, 5 mL of YPD was inoculated with *K. pastoris* and grown overnight at 30 °C at 225 rpm. This culture was diluted 50:1 to start a 50-mL culture that was grown at 30 °C until an $OD_{600\ nm}$ of approximately 1.0 was reached. This culture was then centrifuged (1000×*g*, 5 min) and re-suspended in 9 mL BEDS (10 mM bicine-NaOH, pH 8.3, 3% (*v*/*v*) ethylene glycol, 5% (*v*/*v*) DMSO, 1 M sorbitol) and 1 mL 1.0 M DTT. This solution was shaken for 5 min at 30°, 225 rpm. Cells were then pelleted (1000×*g*, 5 min) and resuspended in 1 mL BEDS. Competent cells were then frozen (−80 °C) in 100 μL aliquots.

For transformation of linear DNA in electro-competent *K. pastoris* cells, approximately 10 μg of plasmid DNA was linearized with either *SacI* or *SpeI* (FastDigest, Thermo Scientific), depending on the construct, for 2–3 h at 37 °C. After digestion, the linear fragment was purified (Cycle Pure Kit, Omega) and eluted in 10 μL. The linear fragment was then mixed with 100 μL of competent cells in a 0.2-cm electroporation cuvette and the mixture was shocked (GenePulser Xcell, BioRad) at 1.5 kV, 25 μF, 200 Ω and immediately resuspended in 1 mL of a 1:1 mixture of YPD and 1 M sorbitol. After incubation at 30 °C for 1 h, 300 μL of cells were plated onto YPDS plates containing 500 μg/mL zeocine (Invitrogen) and incubated at 30 °C.

After colonies were sufficiently grown, generally 2–3 days, colonies were screened for proper integration by colony PCR. Cells were suspended in 10 μL sterile water and patched onto a fresh YPDS plate containing 500 μg/mL zeocine. The suspension was then cycled between 5 min at 95 °C and 10 min at −80 °C three times to sufficiently lyse the cells. This mixture was then used as template DNA in a PCR reaction (GoTaq Hot Start Master Mix, Promega).

Fermentation

One colony that showed a positive amplicon in colony PCR analysis was used to express each of the heparin biosynthetic enzymes. Fermentations were carried out in either 500 mL or

4 L scale. For both scales, seed cultures were grown at 1/10 the final volume in BMGY supplemented with 100 μg/mL zeocine for 36 h at 30 °C, 250 rpm. Before inoculation, the seed cultures were centrifuged (1000×*g*, 5 min) and resuspended in sterile water to get rid of residual undefined growth media. In both cases, small (1–2 mL) samples were taken sporadically to measure cell growth by optical density at 600 nm.

Each fermentation was ran in a 500-mL unit of a parallel DASGIP fermentation system. Briefly, 500 mL of BSM was sterilized in the fermentation vessel. The pH, dissolved oxygen (DO), temperature, and agitation rates were maintained at 5.0, 30%, 30 °C, and 500 rpm, respectively. The seed culture was inoculated into the fermenter such that the initial $OD_{600\ nm}$ was approximately 0.7. Growth in BSM was initially supported by glycerol until the glycerol was consumed after approximately 24 h, as indicated by an upward spike in the DO. Glycerol (50% glycerol supplemented with 12 mL/L trace salt medium) was then fed to the culture at an initial rate of 2 mL/L/h for 60 h to achieve a high cell density of ~100 $OD_{600\ nm}$. Glycerol feeding was then halted for a couple of hours to allow for the consumption of all remaining glycerol, as indicated by another spike in DO. Methanol (100% supplemented with 12 mL/L trace salt medium) was then fed to the culture to induce the expression of heterologous protein. The methanol feed rate was slowly increased such that the cells were not killed by excessive amounts of methanol accumulating in the culture. For the first 5 h, methanol was fed at 0.5 mL/L/h, which increased to 1 mL/L/h for the next 24 h, and finally was increased to 1.5 mL/L/h until the fermentation was finished after approximately 60–75 h of induction.

In the 4 L fermentations, 4 L BSM was autoclaved in a 7-L fermentation vessel and the vessel was connected to the controller (New Brunswick Scientific). The feed and induction was carried out identically to the smaller-scale fermentation, except that it was not automated. The feed was often stopped for a couple of minutes to observe the change in DO to ensure that glycerol and methanol were not accumulating. If the DO rose quickly, there was not an accumulation. If the DO stayed low, then the carbon source was accumulating and the feed rate was lowered until the cells grew adequately.

Purification of protein

After 60–75 h of induction, the methanol feeding was stopped and the cells were allowed to grow until a spike in DO indicated that residual methanol was consumed, generally after 2–5 h. Next, cells were pelleted by centrifugation at 30,000×*g* for 1 h at 4 °C. The pellet was discarded, and the pH of the cell-free media was adjusted to 7.0 with NH_4OH. Precipitate was then removed with another round of centrifugation, and the supernatant was passed through a polyethersulfone 0.22-μm filter (Corning) for further clarification. Expressed his-tagged protein was then purified by immobilized metal ion chromatography (IMAC) using a POROS MC/20 10MMD × 100MML PEEK column packed with nickel resin (Life Technologies). Protein was eluted with 100 mM imidazole and the fraction correlating with a UV peak on the FPLC was removed and buffer exchanged with MES buffer pH 7.0.

Cell-free growth media from 4 L fermentations had to be concentrated to run on the FPLC for protein purification. To this end, after the cells were pelleted as described above, the cell-free media was concentrated approximately 10-fold by ultrafiltration using a 10-kDa centrifugal membrane (Millipore). Purification was then carried out as described above.

Deglycosylation, SDS-PAGE, and Western blot analysis

Prior to analysis by SDS-PAGE, purified protein samples were deglycosylated with Remove-iT PNGase-F (New England Biolabs). Briefly, approximately 30 μg of each of the proteins were denatured at 95 °C for 10 min, followed by incubation with PNGase-F at 37 °C overnight. Following the deglycosylation reaction, the PNGase F was removed from the sample using magnetic beads (New England Biolabs). Protein was then analyzed for purity by running on SDS PAGE using a standard protocol. Western blotting was performed with an antibody against the 6× His-tag (Novagen) using standard protocols.

Sulfotransferase assays

In each reaction, the sulfotransferase was mixed with the sulfo-donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the polysaccharide substrate, and MES buffer pH 7.0. Because the enzymes each have two substrates, kinetic reactions were carried out with varying concentrations of both PAPS and polysaccharide. Reactions were incubated at 37 °C and samples were taken every 6 min for the first 30 min, and then one final time point at 60 min to measure enzyme kinetics. When samples were taken, they were transferred to 100 μL digestion buffer containing 2 μg of trisulfated disaccharide internal standard (Iduron, UK) and incubated at 95 °C for 10 min to stop the enzymatic reaction.

The resulting sulfated polysaccharides were digested by 10 mU each of heparin lyase I, II, and III at 37 °C for 12 h, after which the reaction was filtered through a 3-kDa molecular weight cut-off centrifugal filter (Millipore) to separate the resulting oligosaccharides from the lyases and terminate the digestion. The membrane was washed twice with 100 μL distilled water and the filtrates containing the oligosaccharide products were dried by vacuum centrifugation. The dried samples were AMAC-labeled by adding 10 μL of 0.1 M 2-aminoacridone (AMAC) in dimethylsulfoxide/acetic acid (17/3 ratio) and incubating at room temperature for 10 min.

Next, 10 μL of 1 M $NaBH_3CN$ was added and the reaction was incubated at 45 °C for 1 h. A mixture containing all eight disaccharide standards (25 ng/μL) was labeled the same way. After AMAC-labeling, the samples were centrifuged and the supernatant was recovered.

Labeled disaccharides were then analyzed by LC-MS on an Agilent 1200 LC/MSD instrument equipped with a 6300 ion-trap and a binary pump. Liquid chromatography (LC) was run on an Agilent Poroshell 120 C18 column at 45 °C, with a flow rate of 300 μL/min. The mobile phase consisted of A, 50 mM NH_4Ac in water and B, methanol in the following gradient: 0–10 min, 10–35% B; 10–13 min, 35–100% B; and 13–19 min, 100–10% B. For mass spectrometry (MS), the electrospray was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350 °C. Mass range of the spectrum was 300–900 m/z. Nitrogen (8 L/min 40 psi) was used as a drying and nebulizing gas.

Results

Integration of heparin biosynthetic genes into the genome of *K. pastoris*

Colony PCR analysis was used to verify the integration of the heparin biosynthetic enzyme genes into the genome of *K. pastoris*. Overall the integration efficiency was high (Fig. 1). The integration efficiencies were as follows: 3-OST-1, 6/15; 2-OST, 7/8; 6-OST-1, 6/8; 6-OST-3, 7/8; and 6-OST-3, 6/8.

Expression of the heparin biosynthetic enzymes in *K. pastoris*

Strains of *K. pastoris* each harboring a genomic copy of heparin biosynthetic genes were all able to reach high cell densities in 4 L fermentations (Fig. 2). Each of the strains was able to reach a cell density of at least 30 g/L DCW, while 3-OST-1-expressing strains were capable of reaching nearly 40 g/L DCW.

All four of the heparin biosynthetic enzymes were purified from 4 L *K. pastoris* fermentations, deglycosylated with PNGase F, and run on SDS PAGE to verify their identity and purity (Fig. 3). While all of the enzymes look relatively pure, it is noteworthy that most of the enzymes are running at a lower apparent molecular weight than they are expected to. For instance, the expected molecular weight of His-tagged 6-OST-1 is 42 kDa, but the enzyme runs at an apparent molecular weight of 30 kDa. The overall yields of protein expression were variable, but generally low. The yields of typical 4 L fermentations for each strain are displayed in Table 2. The highest yields obtained are from 3-OST-1, which seems to correlate with its high cell density.

Enzyme kinetic analysis

A comparison of the enzyme kinetics, as measured by LC-MS disaccharide analysis, is presented in Tables 3 and 4. Enzyme kinetics utilized PAPS (Sigma-Aldrich) and three different polysaccharide substrates, completely desulfated N-sulfated heparin (CDNSH), N-sulfoheparosan (NSH) (Bhaskar et al., 2012) and heparan sulfate (Celsus, Cincinnati, OH). The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *K. pastoris* expressed 2-OST were calculated to be 68 pmol/min mg enzyme and 43 μM, respectively (Fig. 4). The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *E. coli* expressed 2-OST were calculated to be 182 pmol/min mg enzyme and 17 μM, respectively. The $V_{max,\ CDNSH}$ and $K_{m,\ CDNSH}$ values of *K. pastoris*-expressed 2-OST were calculated to be 100 pmol/min mg enzyme and 80 μg/mL, respectively. The $V_{max,\ CDNSH}$ and $K_{m,\ CDNSH}$ values of *E. coli* expressed 2-OST were calculated to be 275 pmol/min mg enzyme and 235 μg/mL, respectively.

The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *K. pastoris* expressed 6-OST-1 were calculated to be 23 pmol/min mg enzyme and 15 μM, respectively. The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *E. coli* expressed 6-OST-1 were calculated to be 20 pmol/min mg enzyme and 36 μM, respectively. The $V_{max,\ NSH}$ and

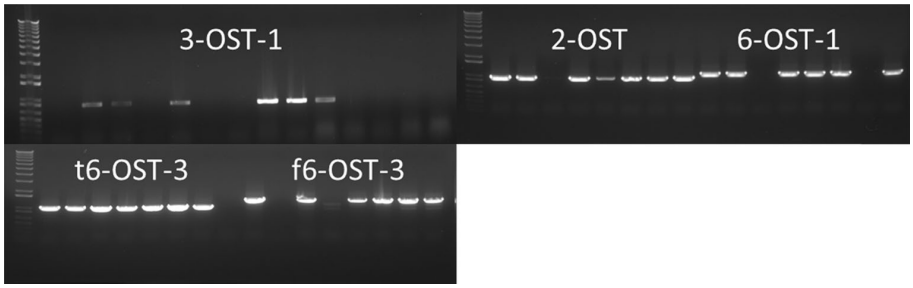

Fig. 1 Colony PCR analysis to verify the integration of the heparin biosynthetic genes into the genome of *K. pastoris*. In each panel, the first lane is 1 kb Plus DNA ladder. Each subsequent lane is colony PCR of one cloned colony. Fifteen colonies were screened for 3-OST-1, and eight colonies were screened for the other constructs

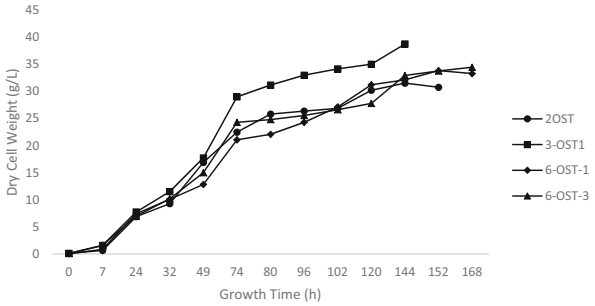

Fig. 2 Growth of *K. pastoris* strains expressing heparin biosynthetic enzymes as a function of time. Each strain grown in individual 4 L fermentation

$K_{m,\ NSH}$ values of *K. pastoris* expressed 6-OST-1 were calculated to be 36 pmol/min mg enzyme and 49 μg/mL, respectively. The $V_{max,\ NSH}$ and $K_{m,\ NSH}$ values of *E. coli* expressed 6-OST-1 were calculated to be 29 pmol/min mg enzyme and 71 μg/mL, respectively.

The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *K. pastoris* expressed 6-OST-3 were calculated to be 102 pmol/min mg enzyme and 11 μM, respectively. The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *E. coli* expressed 6-OST-3 were calculated to be 52 pmol/min mg enzyme and 5 μM, respectively. The $V_{max,\ NSH}$ and $K_{m,\ NSH}$ values of *K. pastoris* expressed 6-OST-3 were calculated to be 95 pmol/min mg enzyme and 18 μg/mL, respectively (Fig. 5). The $V_{max,\ NSH}$ and $K_{m,\ NSH}$ values of *E. coli* expressed 6-OST-3 were calculated to be 130 pmol/min mg enzyme and 15 μg/mL, respectively.

The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *K. pastoris* expressed 3-OST were calculated to be 143 pmol/min mg enzyme and 212 μM, respectively. The $V_{max,\ PAPS}$ and $K_{m,\ PAPS}$ values of *E. coli* expressed 3-OST were calculated to be 103 pmol/min mg enzyme and 144 μM, respectively. The $V_{max,\ HS}$ and

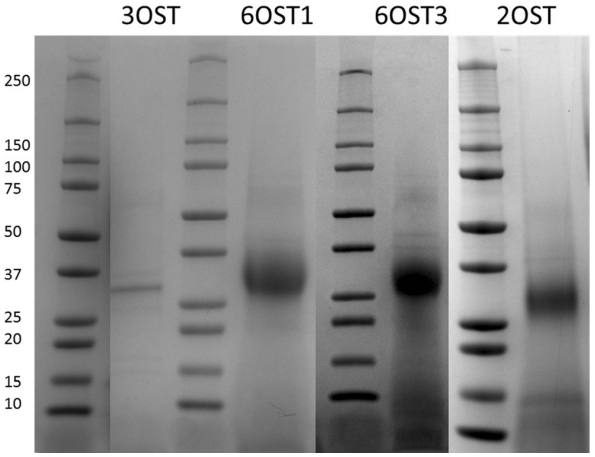

Fig. 3 SDS PAGE of purified heparin biosynthetic enzymes expressed in *K. pastoris* after deglycosylation. 3-OST-1, 6-OST-1, 6-OST-3, C_5-epimerase, and 2-OST were purified using IMAC and then deglycosylated with PNGaseF

Table 2 Yields of purified heparin biosynthetic enzymes expressed from *K. pastoris*

Enzyme	Yield (mg/L)
2-OST	0.65
3-OST-1	1.0
6-OST-1	0.72
6-OST-3	0.68

$K_{m,\ HS}$ values of *K. pastoris* expressed 3-OST were calculated to be 122 pmol/min mg enzyme and 26 μg/mL, respectively. The $V_{max,\ HS}$ and $K_{m,\ HS}$ values of *E. coli* expressed 3-OST were calculated to be 100 pmol/min mg enzyme and 203 μg/mL, respectively.

Discussion

We have successfully expressed four secreted sulfotransferases required for the chemoenzymatic synthesis of heparin using methylotrophic yeast *K. pastoris*. Disaccharide analysis by an LC-MS method showed that His-tagged 2-OST expressed in *K. pastoris* was capable of converting nearly 100% of the NS residues of CDNSH to NS2S residues. Additionally, the *K. pastoris*-expressed 2-OST has a $K_{M\text{-}PAPS}$ that is similar to that of the *E. coli*-expressed enzyme, and a $K_{M\text{-}CDNSH}$ that is approximately twofold lower than the *E. coli*-expressed enzyme, suggesting that the glycosylated enzyme had a higher affinity for its polysaccharide substrate. However, the V_{max} values measured showed that the *E. coli*-expressed enzymes were capable of approximately threefold faster reactions.

When expressed in *K. pastoris*, 6-OST-1 had a slightly lower $K_{m,\ PAPS}$ and a similar $K_{M,\ NSH}$ to the *E. coli*-expressed enzyme. These results indicate that the enzyme has a similar affinity for the GAG substrate, regardless of glycosylation. The enzymes also have similar V_{max} values.

The 6-OST-3 expressed in *K. pastoris* was also able to convert nearly 100% of the NS residues in NSH to NS6S. Kinetic analysis showed that glycosylated 6-OST-3 had similar $K_{M,\ PAPS}$ and $K_{M,\ NSH}$ to the non-glycosylated enzyme.

Table 3 Comparison of Km values for sulfotransferases expressed in *E. coli* and K. pastoris

	2-OST		6-OST-1		6-OST-3		3-OST-1	
	$K_{m,\ PAPS}$ (μM)	$K_{m,\ CDNSH}$ (μg/mL)	$K_{m,\ PAPS}$ (μM)	$K_{m,\ NSH}$ (μg/mL)	$K_{m,\ PAPS}$ (μM)	$K_{m,\ NSH}$ (μg/mL)	$K_{m,\ PAPS}$ (μM)	$K_{m,\ NSH}$ (μg/mL)
E. coli	17	235	36	71	5	15	144	203
K. pastoris	43	80	15	49	11	18	212	26

Table 4 Comparison of Vmax for sulfotransferases expressed in *E. coli* and *K. pastoris*

	2-OST		6-OST-1		6-OST-3		3-OST-1	
	$V_{max,\ PAPS}$ (pmol/min)	$V_{max,\ CDNSH}$ (pmol/min)	$V_{max,\ PAPS}$ (pmol/min)	$V_{max,\ NSH}$ (pmol/min)	$V_{max,\ PAPS}$ (pmol/min)	$V_{max,\ NSH}$ (pmol/min)	$V_{max,\ PAPS}$ (pmol/min)	$V_{max,\ NSH}$ (pmol/min)
E. coli	182	275	20	29	52	130	103	100
K. pastoris	68	100	23	36	102	95	143	122

The glycosylated and non-glycosylated forms of 3-OST-1, prepared in *K. pastoris* and *E. coli*, respectively, show similar $K_{M,\ PAPS}$, but the glycosylated enzyme was shown to have a $K_{M,\ HS}$ that is approximately 10-fold lower, indicating a much stronger affinity for that substrate. The two enzymes showed very similar V_{max} values.

Together, the findings here show that *K. pastoris* may be a suitable microbial host for the expression of the sulfotransferases required for the biosynthesis of non-animal-sourced heparin. High sulfotransferase activities were observed for 3-OST-1, 2-OST, 6-OST-1, and 6-OST-3. The levels of protein expression were low, although this is not uncommon for protein expression in *K. pastoris* (Ahmad et al. 2014). It is possible likely that yield could be increased by fermentation or strain optimization.

The low yield of enzymes from *K. pastoris* is one of the biggest problems associated with its use for enzyme expression. A typical 4 L fermentation yields only ~2 mg of purified protein. In contrast, the *E. coli* expression system generally affords at least 5 mg per liter. However, multiple studies have described approaches to increase the yield of protein expression from *K. pastoris*. One place to begin optimization would be changing the strain of *K. pastoris* used for expression. In regard to the *aox1* gene, which shares a promoter with the proteins of interest that are integrated into the genome, there are three phenotypes possible: Mut^+, Mut^-, and Mut^S (Cereghino and Cregg 2000). The Mut^+ phenotype describes strains with wild type copies of the *aox1* gene that are capable of expressing AOX1 in the presence of methanol. These cells can grow using methanol as the sole carbon source. The Mut^S phenotype describes strains that have a nonfunctional copy of *aox1* but still can express *aox2* in the presence of methanol. Expression of AOX is weaker here because of the weaker promoter that regulates *aox2* expression. Due to lower expression of AOX, these strains are less tolerant of methanol, so the feed rate of methanol has to be decreased. The Mut^- phenotype describes strains, which do not express either *aox1* or *aox2*. As a result, these cells cannot utilize methanol as a carbon source and must grow on glycerol or carbon to permit growth. Multiple studies have shown that, while the growth

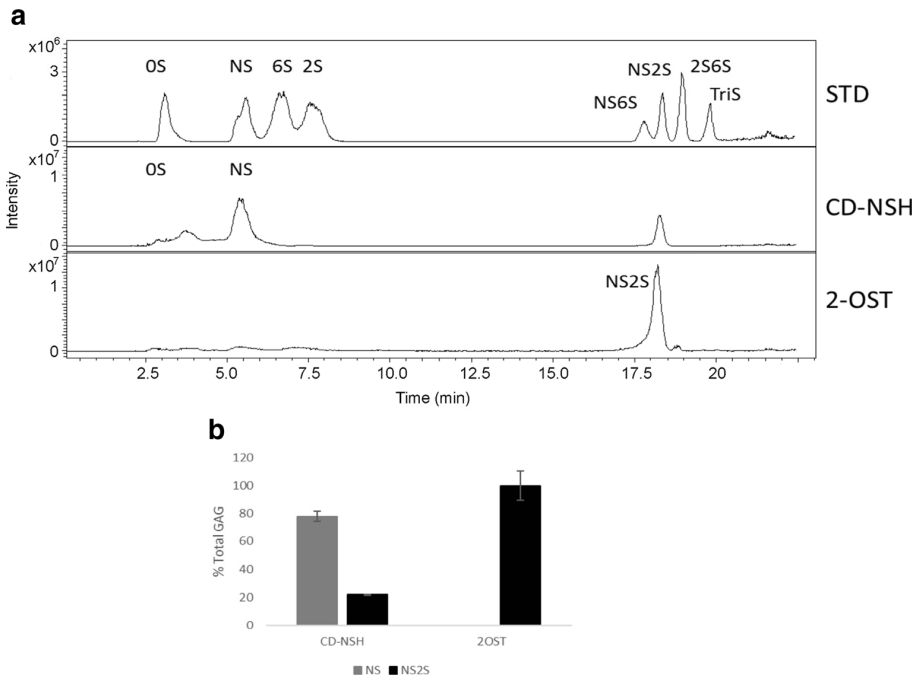

Fig. 4 LC-MS analysis of the sulfotransferase activity of *K. pastoris*-expressed 2-OST on CD-NSH. LC-MS spectrum comparing peaks found in negative control (CD-NSH), and 2-OST treated CD-NSH (**a**). Percent GAG calculated based on peak areas (**b**). Standard (STD) contained eight known disaccharides. *Error bars* are standard deviation of duplicate runs

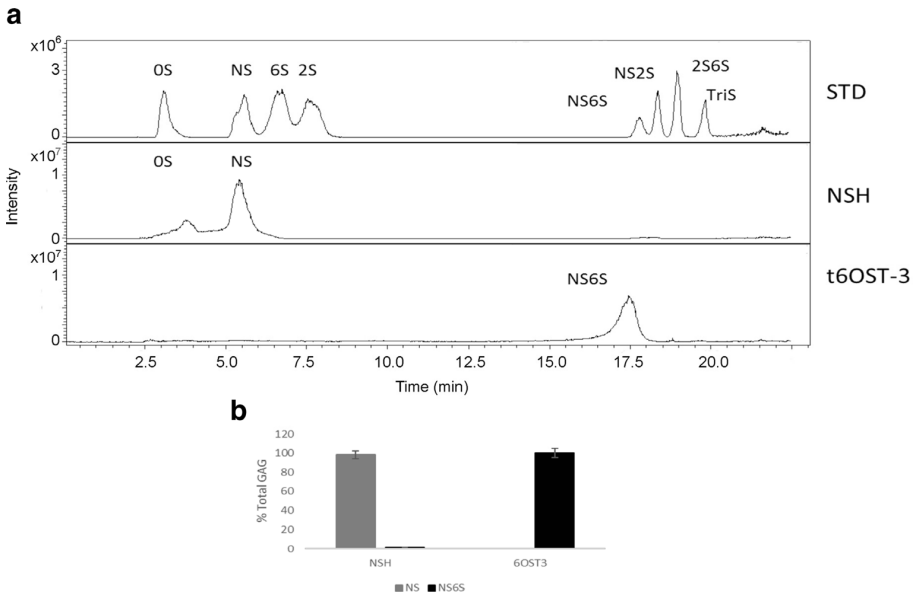

Fig. 5 LC-MS disaccharide analysis of t6-OST-3 sulfotransferase activity. Standard consists of eight known disaccharides. *Error bars* are standard deviation of duplicate runs

rate of Mut^S strains are lower than Mut$^+$ strains, they can show increased protein production (Pla et al. 2006; Orman et al. 2009). Thus, expression of the heparin biosynthetic enzymes in a Mut^S strain may result in increased protein production. Additionally, many studies have shown that one of the most important factors for high yield protein production in *K. pastoris* is the copy number of integrated genes (Clare et al. 1991; Sha et al. 2013; Yang et al. 2016). Though it is noteworthy that some researchers saw a decrease in expression with increasing copy number (Yang et al. 2012), increased copy number in *K. pastoris* is usually obtained by selecting for transformants on increased concentrations of antibiotics. This could be an easy solution to a large problem.

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

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