

High cell density cultivation of a recombinant *E. coli* strain expressing a key enzyme in bioengineered heparin production

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Abstract A bioengineered heparin, as a replacement for animal-derived heparin, is under development that relies on the fermentative production of heparosan by *Escherichia coli* K5 and its subsequent chemoenzymatic modification using biosynthetic enzymes. A critical enzyme in this pathway is the mammalian 6-*O*-sulfotransferase (6-OST-1) which specifically sulfonates the glucosamine residue in a heparin precursor. This mammalian enzyme, previously cloned and expressed in *E. coli*, is required in kilogram amounts if an industrial process for bioengineered heparin

is to be established. In this study, high cell density cultivation techniques were exploited to obtain recombinant 6-OST-1. Physiological studies were performed in shake flasks to establish optimized growth and production conditions. Induction strategies were tested in fed-batch experiments to improve yield and productivity. High cell density cultivation in 7-l culture, together with a coupled inducer strategy using isopropyl β -D-1-thiogalactopyranoside and galactose, afforded 482 mg l⁻¹ of enzyme with a biomass yield of 16.2 mg g_{cdw}⁻¹ and a productivity of 10.5 mg l⁻¹ h⁻¹.

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Introduction

Heparin, a widely used blood anticoagulant, is a sulfated linear polysaccharide prepared from porcine intestine (Linhardt 2003). In 2008, a heparin crisis took place as the result of the adulteration of heparin (Guerrini et al. 2008; Liu et al. 2009), leading our laboratory to consider whether it was possible to prepare a potentially safer bioengineered version of this critical drug (Linhardt and Liu 2012). This process begins with: (1) upstream heparosan prepared from the fermentation of *Escherichia coli* K5 (Wang et al. 2010, 2011a), (2) midstream chemical modification of heparosan to form the intermediate *N*-sulfo- and *N*-acetyl-heparosan (Wang et al. 2011b), and (3) downstream treatment with enzymes and cofactors to obtain bioengineered heparin (Zhang et al. 2008; Wang et al. 2011c). This process has been successfully applied to the preparation of small amounts of bioengineered heparin (Zhang et al. 2008; Wang et al. 2011c) that closely resembles porcine intestinal heparin in both structure and activity (Lindahl et al. 2005; Zhang et al. 2008, 2011; Wang et al. 2011c). In an effort to prepare kilogram amounts of

bioengineered heparin required for preclinical evaluation, this process must be scaled-up. The upstream production of kilogram quantities of heparosan has been accomplished and the midstream chemical steps have been shown to be successful on the gram scale (Wang et al. 2011a, c). We have now turned our attention to the downstream enzymatic process and the preparation of larger quantities of the recombinant sulfotransferases required.

The current study focuses on recombinant 6-*O*-sulfotransferase-1 (6-OST-1) enzyme of mammalian origin, that has up to now only been produced in shake flask culture in Luria Broth (LB) media by an engineered *E. coli* strain with isopropyl β -D-1-thiogalactopyranoside (IPTG) induction affording final cell culture densities of 1 g_{cdw}/l and 6-OST-1 in amounts of <10 mg/l (Chen et al. 2005). High cell density cultivations are often used for efficient production of recombinant enzymes having the added advantages of improved yield and productivity of the process as well as increased cost-effectiveness (Shiloach and Fass 2005; Lee 1996). Research on high cell density cultivation for recombinant protein production includes the formulation of media suitable for both growth and protein expression, the investigation of the optimal culture and induction conditions, and the study of the best nutrient feeding and aeration strategies to avoid inhibiting by-product formation (Shiloach and Fass 2005). Glycerol-based media are frequently used in high cell density cultivation to finely modulate the carbon source uptake by the bacterial cells and to limit overflow metabolism effects (Shiloach and Fass 2005; Restaino et al. 2011). High biomass concentrations are achieved in fed-batch fermentations with a strict control of growth parameters and avoiding the formation of high concentrations (10 g/l) of acetic acid that could inhibit the cell growth and block the enzyme expression (Shiloach and Fass 2005). High biomass levels must be reached before induction to have sufficient cells to produce the large amounts of enzymes required for industrial processes. A further complicating factor is that induction usually slows cell growth especially, as in the current case, when it is performed at a reduced temperature required for the optimal expression of active enzyme rather than for the optimal production of biomass. High biomass concentrations are usually achieved in fed batch in a well-controlled fermentor to avoid the formation of by-products, such as acetic acid. High concentrations (10 g/l) of acetic acid inhibit the cell growth and block the enzyme expression. An appropriate feeding schedule and maintaining adequate dissolved oxygen levels through adequate agitation and sparging is often used to avoid acetic acid buildup in the medium (De Mey et al. 2007). While a too high concentration of carbon source leads to acetic acid formation, too low levels of carbon source concentrations can result in cell starvation, resulting in cell stress favoring the propagation of the plasmid-free population, lowering enzyme production.

Thus, a balance between the necessity to reach high cell density and to control the acetate formation is needed for the successful production of recombinant enzymes. Furthermore, the type and concentration of inducer and the time of induction must be optimized. Sufficient inducer is required to be certain that all the cells are effectively induced but not so much as to result in toxic effects of the inducer on the cell growth, such as can be the case using IPTG. Because of the metabolic burden effect, high inducer concentrations do not automatically lead to the maximal protein expression and the optimal inducer concentration is determined balancing the decrease of biomass formation, after induction, with the increase in protein production (Donovan et al. 1996). Typically, IPTG concentrations higher than 1.0 mM should be avoided and induction can be further enhanced through the concurrent use of a second inducer (Weng et al. 2006; Carvalho et al. 2012).

Materials and methods

Materials

All media components, fermentation reagents, chemicals used for preparation of biomass extraction buffer, and chemicals and standards used in HPLC analysis, were from Sigma-Aldrich (USA). LB broth powder was purchased from BD Biosciences (USA). Carbenicillin, tetracycline hydrochloride, and IPTG were from Gold Biotechnology (USA). Kanamycin sulfate was purchased from GIBCO (USA).

Bacterial strain and media

The recombinant *E. coli* strain expressing 6-OST-1 maltose-binding protein fusion product was prepared by inserting the plasmid pMalc2x expressing 6-OST-1 into Rosetta-gami B (DE3) cells (Novagen, USA) (Chen et al. 2005). This strain was stored frozen at -80°C in stock solutions of LB medium containing 20 % (v/v) glycerol. Shake flask experiments were performed in LB medium and in a new formulated glycerol-based medium (20.0 g l⁻¹ glycerol, 5.0 g l⁻¹ soya peptone, 0.3 g l⁻¹ thiamine, 13.5 g l⁻¹ KH₂PO₄, 4.0 g l⁻¹ (NH₄)₂HPO₄, 1.4 g l⁻¹ MgSO₄·7H₂O, 1.7 g l⁻¹ citric acid, and 10.0 ml l⁻¹ of a trace metal solution consisting 10.0 g l⁻¹ FeSO₄·7H₂O, 2.0 g l⁻¹ CaCl₂, 2.2 g l⁻¹ ZnSO₄·7H₂O, 0.5 g l⁻¹ MnSO₄·4H₂O, 1.0 g l⁻¹ CuSO₄·5H₂O, 0.1 g l⁻¹ (NH₄)₆MoO₂₄·4H₂O, and 0.02 g l⁻¹ Na₂B₄O₇·10H₂O dissolved in 5 M HCl); after autoclaving, the pH 7 glycerol+soya peptone medium was used also for fed-batch fermentation experiments after in situ sterilization. In both shake flask and fed-batch experiments, salts and trace metals were added to the sterile medium after filtration through a 0.22- μm

membrane (Millipore, France). Carbenicillin (50 mg l⁻¹), tetracycline (12.5 mg l⁻¹), and kanamycin (15 mg l⁻¹) were always added to the media for both growth and propagation of the strain.

Shake flask experiments

Shake flask experiments were performed in a rotary air shaker (C24 Incubator, New Brunswick Scientific, USA), at 220 rpm and at 37 °C, using 100 ml of medium in 250 ml baffle-equipped shake flasks. A 10-ml culture tube was inoculated with the bacterial glycerol stock solution and grown overnight on the same medium then used as seed for each experiment. The biomass increase was checked by measuring the absorbance at 600 nm with a spectrophotometer (UV-mini 1240, Shimadzu, Japan) and by determining the cell dry weight of 2 ml broth samples after centrifugation at 10,800×g (Minispin plus, Eppendorf, USA) and drying in an oven (Isotemp 500 series, Thermo Fisher Scientific, USA) at 60 °C for 18 h. Induction was performed when the biomass concentration was around 0.3–0.35 g_{cdw} l⁻¹, after that the growth temperature was reduced to 25 °C. Preliminary experiments were performed in duplicate in LB medium or on the glycerol+soya peptone medium, inducing with 0.2 mM IPTG, to compare both growth and enzyme production. Control experiments were performed in parallel on the same media without any induction. Further experiments were performed in the glycerol+soya peptone medium by testing different inducers (0.2 mM IPTG, 0.2 mM IPTG+28 mM galactose (Gal), or 28 mM Gal). In all cases, the final biomass was harvested at 26 h of growth, after 21–22 h of induction, by centrifuging the broth at 4 °C and 3,220×g for 30 min (Centrifuge 5810 R, Eppendorf, USA). The centrifuged biomass was frozen at -80 °C.

Fed-batch experiments

A 15-l bioreactor (Bio-Flow 4500, New Brunswick Scientific, USA), in situ sterilizable, equipped with pH and pO₂ probes (Mettler Toledo, Switzerland) and three peristaltic pumps for addition of acid, base, and feeding solutions were used for fed-batch experiments. During growth, the fermentation parameters were controlled and data were collected by BioXpert V1.5 software (Applikon Biotechnology, the Netherlands). The pO₂ electrode was calibrated using a pure oxygen flow as the 100 % value. Fed-batch experiments were performed at 37 °C and pH of 7.0 in the glycerol+soya peptone (7-l), after inoculation with 0.5 l of seed grown overnight in 1-l shake flask in the same medium. After 12–14 h of batch phase, the culture was fed with a concentrated solution (250 g l⁻¹ glycerol, 60.0 g l⁻¹ soya peptone, 13.5 g l⁻¹ KH₂PO₄, and 5.7 g l⁻¹ MgSO₄·7H₂O). In a first fed-batch experiment (FB A), the culture was constantly fed

for 18 h at a rate of 3.9 g l⁻¹ h⁻¹, while in the other two fed-batch experiments (FB B and FB C), a feed rate in the range between 2.9 and 3.2 g l⁻¹ h⁻¹ was used until induction; after that, the feeding was slowed to 0.9–1.2 g l⁻¹ h⁻¹ to avoid any over-flow metabolism effect and acetic acid formation. Induction was performed after setting the temperature at 25 °C by using 1.0 mM IPTG (FB A) or 1.0 mM IPTG+28 mM Gal (FB B and C) at around 15 (FB A and B) or 24 g_{cdw} l⁻¹ (FB C). During the entire process, the pO₂ value inside the fermentor was maintained at >10 % by modulating the stirring and the airflow values in the range from 450 to 700 rpm and 20 to 30 l min⁻¹, respectively. The pH was maintained at 7.0 by addition of (29 %, v/v) NH₄OH and/or (37 %, v/v) HCl. Culture samples (25 ml) were regularly withdrawn to check the cell growth by measuring the absorbance at 600 nm and by determining the cells dry weight, as already described. The samples were then centrifuged at 3,220×g and 4 °C for 30 min (Centrifuge 5810 R, Eppendorf, USA). The supernatants and the biomass pellets were separated and frozen at -80 °C. The final biomass was harvested after 18–20 h of induction by centrifugation (Sorvall Evolution RC, Thermo Fisher Scientific, USA) of the broth at 4 °C and 3,500×g for 45 min, and then frozen at -80 °C.

Biomass extraction and enzyme purification

Biomass pellets coming from shake flask and fed-batch growth were resuspended in 10 and 30 ml, respectively, of an extraction buffer (25 mM Tris and 200 mM NaCl, (pH 7.4)) and then sonicated (Sonicator 3000, Misomix, USA) in ice for 5 min (10 s on and 10 s off; power level, 6.0). The sonicated samples were centrifuged at 4 °C and at 3,220×g for 30 min. Crude extract (4 ml) was purified at 4 °C by loading them on a 4-ml bed amylose resin-packed column (New England, Biolabs, USA), equilibrated with the extraction buffer. In case of crude extract coming from fermentation experiments, the loaded sample was first diluted (1:2) with the same buffer. The loaded resin was shaken (Innova 3000, New Brunswick Scientific, USA) at 4 °C and 75 rpm for 1.5 h. After that the flow through was collected and a wash step was performed by using three column volumes of wash buffer and by shaking the column for 15 min. Elution of the 6-OST-1 MBP fusion protein was performed with one column volume of 25 mM Tris, 200 mM NaCl, and 40 mM maltose elution buffer (pH 7.4) after shaking for 30 min. The eluted fraction, containing the purified enzyme, was used for SDS-PAGE, protein content assays, activity, and Western blot analyses.

SDS PAGE and protein concentration analysis

The crude extract or the purified enzyme samples, coming from both shake flask and fed-batch fermentation, were

concentrated by applying 0.5 ml to a 3-kDa spin column (YM-3, Millipore, France) by centrifugation at 4 °C and at 16,000×g (Centrifuge 5810 R, Eppendorf, USA). SDS-PAGE protein analysis was performed on these concentrated samples in a Mini-Protean Tetra system (BIORAD, USA), by loading 50 µl on a 4–15 % precast gel (Mini protean TGX gels, BIORAD, USA) and running in a Tris, glycine and SDS buffer (10× Tris/glycine/SDS buffer, BIORAD, USA) at 100 V for 1.5 h. A 250- to 10-kDa protein standard (Precision Plus Protein Kaleidoscope, BIORAD, USA) was used as ladder. Gels were stained by soaking for 3 h in Coomassie blue solution (Gel Code Blue Safe Protein Stain, Thermo Fisher Scientific, USA). The protein content of both crude extract and purified enzyme samples was determined, in triplicate, by using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions, after a concentration and a buffer exchange step in PBS buffer on spin columns.

Activity assay and mass analysis

The activity of the purified enzyme samples, coming from each of the three fed-batch fermentation experiments, were analyzed by incubating 5.0 µg of purified 6-OST-1 with 2.5 µg of *N*-sulfoheparosan decasaccharide and 2 µg 3'-phosphoadenosine-5'-phosphosulfate (PAPS) in 22.5 µl of 50 mM MES buffer pH 7.0. Initial studies were performed to determine the optimal reaction time to ensure that the assay occurred during the linear initial burst of product production. Based on these studies, a 24-h time point was selected and the reaction was quenched by adding 50 µl of 100 % acetonitrile. The products were then analyzed by hydrophilic interaction chromatography column (2.0×50 mm, Phenomenex, Torrance, CA) coupled with electrospray ionization LTQ-Orbitrap XL FTMS (Thermo Fisher Scientific, San Jose, CA). Mobile phase A was 5 mM ammonium acetate prepared with HPLC-grade water. Mobile B was 5 mM ammonium acetate prepared in 98 % HPLC-

grade acetonitrile with 2 % of HPLC grade water. Agilent 1200 HPLC binary pump was used to deliver the gradient from 10 to 80 % A over 8 min at a flow rate of 250 µL/min after injecting the samples. The optimized MS parameters, used to prevent in-source fragmentation, included a spray voltage of 4.2 kV, a capillary voltage of -40 V, a tube lens voltage of -50 V, a capillary temperature of 275 °C, a sheath flow rate of 30, and an auxiliary gas flow rate of 6. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All FT mass spectra were acquired at a resolution 60,000 within 400–2,000 Da mass range. The substrate peak at m/z 694.08 ($[M-3H]^{3-}$, $z=-3$, MW 2,085.2886) disappeared over time with the appearance of a mono-6-*O*-sulfo-*N*-sulfoheparosan decasaccharide product peak at m/z 720.739 ($[M-3H]^{3-}$, $z=-3$, MW 2,165.2454). The appearance of product was used to calculate specific activity in units of picomoles of product formed per minute per milligram protein.

Western analysis

Western blot analysis was performed on the fed-batch purified enzyme samples. Approximately 40 µg of total protein was loaded and separated on 4–20 % gradient polyacrylamide gel. After transfer to a PVDF membrane, proteins of interest were detected using primary antibody (anti-Hs6st1 Santa Cruz Biotechnology INC., Santa Cruz, CA) and HRP-conjugated secondary antibodies followed by chemiluminescent (Pierce, Super SignalWest Pico ECL substrate) exposure on high performance chemiluminescence film (GE Healthcare, Little Chalfont, UK, Amersham Hyperfilm ECL).

HPLC analysis

Concentrations of glycerol, galactose, and acetate in the medium during the fermentation were measured by HPLC (CBM-20A, Shimadzu, Japan), in duplicate, by using an ion-

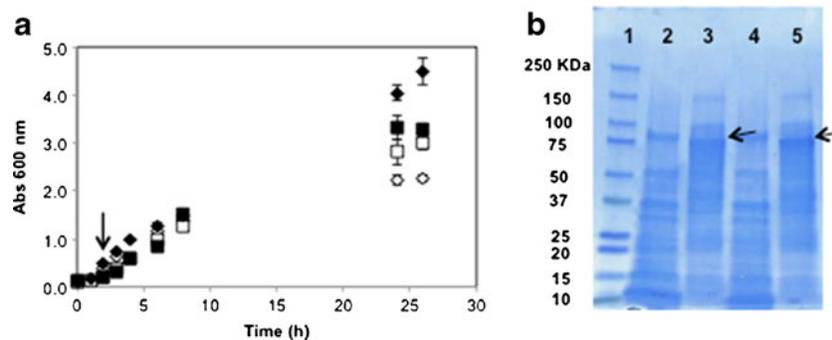


Fig. 1 *E. coli* 6-OST-1 shake flask growth in different media: **a** 26-h growth curves on induced LB (empty diamonds); not induced LB (filled diamonds); induced glycerol and soya peptone (empty squares); and not induced glycerol and soya peptone (filled squares). Induction was performed with 0.2 mM IPTG at 0.6 Abs_{600nm}, as indicated by the

arrow. **b** SDS-PAGE analysis of crude extracts; lanes 1, 250–10 kDa ladder; 2, not induced LB, 3, induced LB; 4, not induced glycerol and soya peptone; and 5, induced glycerol+soya peptone. 6-OST-1 bands are indicated in lanes 3 and 5 by arrows

exchange column (Supelcogel C-610H, 30 cm×7.8 mm, Supelco, USA) at 30 °C and with a 0.1 % (v/v) H₃PO₄ buffer at the flow rate of 0.5 ml/min. Glycerol and galactose were detected by RI (RID-10A, Shimadzu, Japan), while acetate was detected by UV (SPD-20A, Shimadzu, Japan) at 210 nm and integrated using LC solution software by Shimadzu (Japan).

Results

Shake flask experiments

Physiological studies were initially performed in shake flasks to formulate an alternative medium to replace LB medium in fed-batch experiments. This alternative medium must sustain both bacterial growth and enzyme production at high cell densities and be suitable for scaling-up the fermentation process. Shake flask experiments with (or without) induction in LB medium and in glycerol+soya peptone medium were used to test the influence of the effect of medium on enzyme expression and the effect of induction on cell growth. In the first 4 h of the fermentation, before reaching the induction point, growth in LB medium (specific growth rate (μ)=0.503±0.016) was faster than in glycerol+soya peptone medium (μ =0.450±0.002 h⁻¹) (Fig. 1a). As a consequence, the final cell density in the noninduced fermentations reached values of 2.43±0.15 and 1.70±0.05 g_{cdw}l⁻¹, on LB and on glycerol+soya peptone media, respectively. When the culture was induced, cell growth in both media slowed, but a higher final cell density

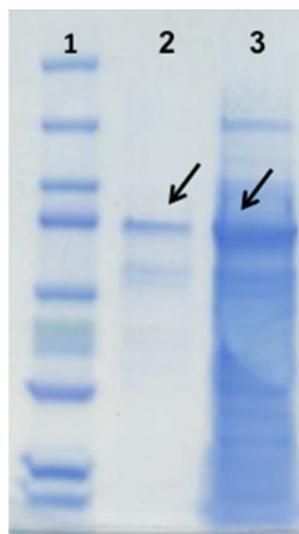


Fig. 2 SDS-PAGE analysis of 6-OST-1 enzyme obtained by extraction and purification of biomass crude extract after shake flask growth in glycerol and soya peptone; lanes 1, 250–10 kDa ladder as reported in Fig. 1; 2, purified 6-OST-1 enzyme (indicated by an arrow); and 3, crude extract with 6-OST-1 enzyme (indicated by an arrow). The identity of the band at 75 kDa in lane 2 was confirmed by Western blot analysis (data not shown)

Table 1 Comparison of concentration and yield values of the purified 6-OST-1 enzyme obtained from 0.2 mM IPTG-induced cell biomass after 26 h of growth in LB and glycerol+soya peptone media

	LB	Glycerol+soya peptone
Purified protein (mg l ⁻¹)	9.85±1.06	9.80±1.20
Biomass (g _{cdw} l ⁻¹)	1.17±0.03	1.56±0.07
Purified protein yield (mg g _{cdw} ⁻¹)	8.38±0.90	6.28±0.82

was observed on glycerol+soya peptone medium (1.56±0.07 g_{cdw}l⁻¹) than in the LB medium (1.17±0.03). Both media resulted in expression of 6-OST-1 as shown by PAGE analysis of crude extract samples (Fig. 1b). The purified enzyme concentration was also similar in both media, although the amount of purified 6-OST-1 per cell mass was higher in LB medium (Fig. 2; Table 1). Glycerol- and soya-based medium was selected to be used in scaled-up fed-batch fermentation because it was capable of sustaining good growth even after induction and afforded 6-OST-1 production comparable to that obtained in LB medium. Furthermore, in contrast to LB medium, this medium used glycerol as a carbon source and contained no animal derived nutrients. Glycerol and soya based medium was suitable to design an industrial fermentation process and afforded an appropriate feeding profile for high cell density cultivations.

Fed-batch experiments

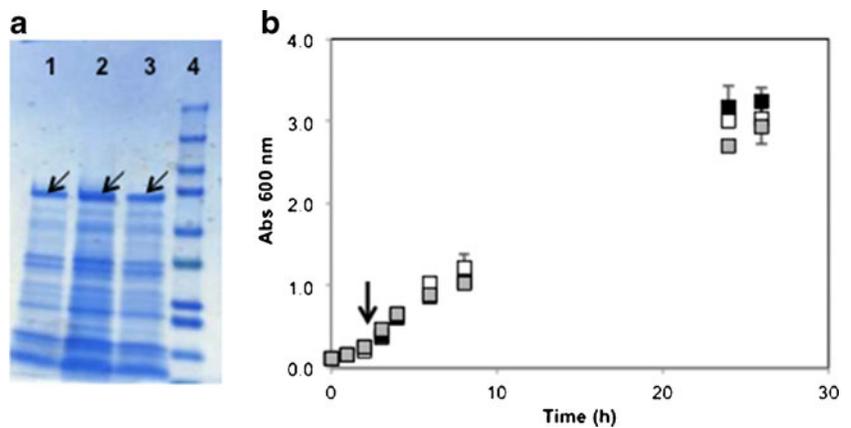
IPTG induction

The glycerol and soya peptone-based medium was used for the 7-l fed-batch experiments to improve the productivity

Table 2 Comparison of three fed-batch fermentation experiments induced with different strategies; with different inducers (FB A and FB B) or at different time points (FB B and FB C)

	FB A	FB B	FB C
Total hours of induction (h)	22.0	21.0	16.0
Inducer (mM)	1.0 mM IPTG	1.0 mM IPTG+ 28 mM Gal	1.0 mM IPTG+ 28 mM Gal
Cell density at induction (g _{cdw} l ⁻¹)	17.4	16.2	22.6
Fermentation time (h)	46.0	45.0	46.0
Maximum cell density (g _{cdw} l ⁻¹)	32.7	28.2	29.8
Purified 6-OST-1 concentration (mg l ⁻¹)	92.8	394.0	482.3
Yield 6-OST-1/biomass (mg g _{cdw} ⁻¹)	2.8	14.0	16.2
Productivity 6-OST-1 (mg l ⁻¹ h ⁻¹)	2.1	8.7	10.5
Final specific activity (pmol min ⁻¹ mg ⁻¹)	82.0	24.0	32.0

Fig. 3 *E. coli* 6-OST-1 shake flask growth in glycerol+soya peptone medium using different inducers. **a** SDS-PAGE analysis of crude extracts; lanes 1, IPTG; 2, IPTG and Gal; 3, Gal LB; 4, 250–10 kDa ladder as reported in Fig. 1; **b** 26-h growth curves induced with IPTG (□), IPTG and Gal (▣), and Gal (■). Induction was performed with 0.2 mM IPTG at 0.6 Abs_{600nm}, as indicated by the arrow



and yield of 6-OST-1. Three experiments were performed to examine the effect of inducer and induction time on enzyme yield. In the first, involving fed batch experiment (FB A), the culture was induced with 1.0 mM IPTG at a cell density of 17.4 g_{cdw} l⁻¹ (Table 2). A higher IPTG concentration was used, compared with 0.2 mM IPTG used in the shake flask experiments, because of the higher cell density at the induction time. After 46 h of growth, 22 h post-induction, the biomass reached 32.8 g_{cdw} l⁻¹, 21-fold higher than observed in shake flasks. At harvest, the cell density was 2.8 mg l⁻¹ h⁻¹ and 92.8 mg l⁻¹ of 6-OST-1 was obtained. Although a 10-fold increase in 6-OST-1 was observed compared with shake flask experiments, the increase was half that of the 21-fold increase in biomass suggesting that 1.0 mM IPTG was insufficient for full induction of 17.4 g_{cdw} l⁻¹ of cells.

IPTG and Gal coupled induction

A coupled induction strategy was next investigated in shake flask experiments, testing the simultaneous use of IPTG and Gal. When used singularly, as alternative to IPTG, Gal results in higher enzyme expression than IPTG alone (Fig. 3a; Table 3) in terms of both production and yield. But, in combination with IPTG, Gal doubled the concentration and yield of 6-OST-1. In addition, coupled induction did not significantly slow the growth or reduce the final biomass value (Fig. 3b). This coupled induction strategy was next tested in the second fed-batch experiment (FB B)

Table 3 Comparison of concentration and yield values of the purified 6-OST-1 enzyme obtained from 0.2 mM IPTG, 28 mM Gal or 0.2 mM IPTG+28 mM Gal-induced cell density after 26 h of growth in glycerol+soya peptone medium

	IPTG	IPTG and Gal	Gal
Purified protein (mg l ⁻¹)	9.90±0.14	17.61±0.54	15.86±1.69
Cell density (g _{cdw} l ⁻¹)	1.57±0.01	1.53±0.12	1.68±0.09
Purified protein yield (mg g _{cdw} ⁻¹)	6.29±0.09	11.50±0.35	9.44±1.00

in which the biomass was induced at the same time point as it was in FB A (Fig. 4a). The final biomass obtained was similar but the enzyme expression was enhanced, both production and yield almost quadrupled reaching values of

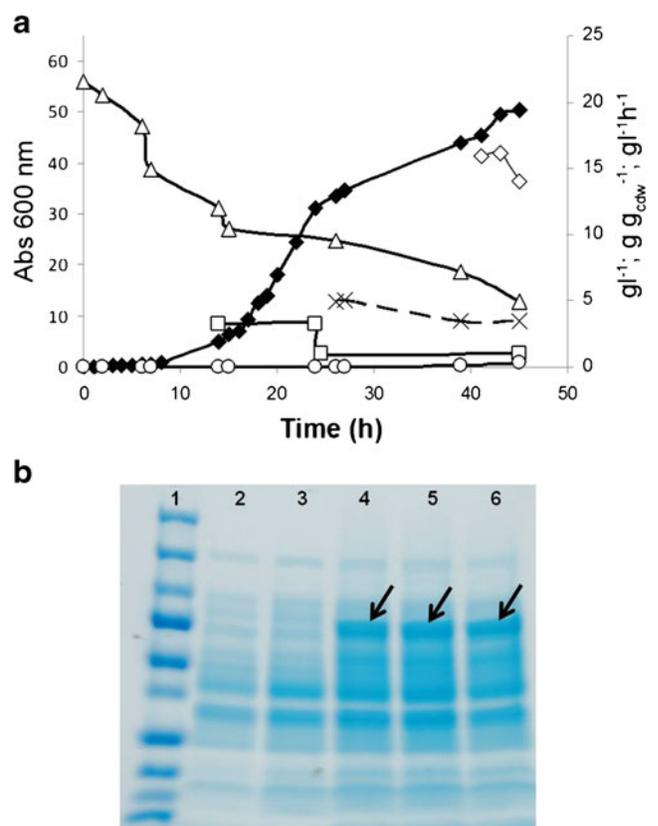


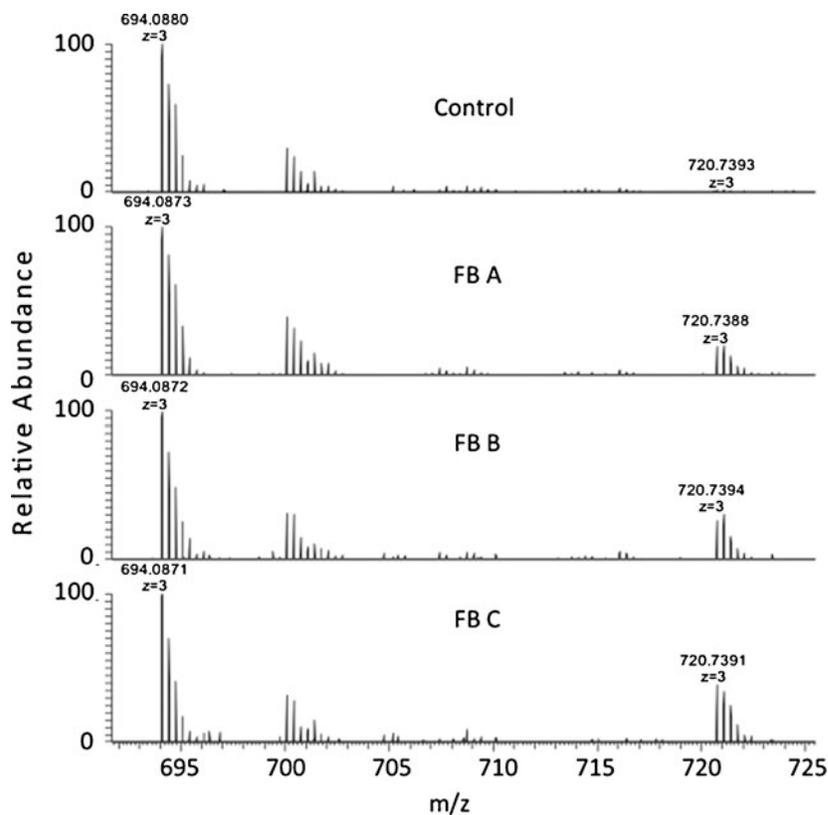
Fig. 4 *E. coli* 6-OST-1 fed batch fermentation in glycerol and soya peptone medium: **a** bacterial growth (filled diamonds), glycerol consumption (empty triangles), feeding profile (empty squares), acetate formation (empty circles), galactose residual concentration (error marks), and protein yield on biomass (empty diamonds). Induction was performed with 0.1 mM IPTG and 28 mM Gal at 31 Abs_{600nm}, as indicated by the arrow. **b** SDS PAGE analysis of crude extracts; lanes 1, ladder as reported in Fig. 1; 2, 24 h of growth, pre-induction; 3, 26 h of growth, 2 h post-induction; 4, 39 h of growth, 14 h post-induction; 5, 41 h of growth, 16 h post-induction; and 6, 45 h of growth, 20 h post-induction. 6-OST-1 bands are visible in lanes 4, 5, and 6 as indicated by the arrows

394 mg l⁻¹ and 14.0 mg g_{cdw}⁻¹, respectively, with a productivity of 8.7 mg l⁻¹ h⁻¹ (Fig. 4b; Table 2). Good control of agitation and aeration conditions also avoided acetate formation in FB B. The coupled induction strategy was again examined in a third fed-batch experiment (FB C), inducing at an even higher cell density of 24 g_{cdw} l⁻¹, and in this case, an enzyme concentration, yield, and productivity of 482 mg l⁻¹, 16.2 mg g_{cdw}⁻¹, and 10.5 mg l⁻¹ h⁻¹, respectively, were achieved (Table 2).

Enzyme activity measurements

The specific activity of 6-OST-1 obtained from the three batch fermentations was next examined using a newly developed sulfotransferase assay. Sulfotransferase assays generally either involve the use of ³⁵S PAPS cofactor or are indirect involving a coupled assay (Paul et al. 2012). In this study, we decided to test a newly developed assay that relies on Fourier transform electrospray ionization mass spectrometry to directly monitor substrate disappearance and product appearance (Fig. 5). This assay offers several improvements over older methods as it does not require radiolabel, does not result in false positives, and provides information on how many sulfo groups are transferred to a given substrate. The specific activity of 6-OST-1 prepared in FB A (induced by IPTG) was up to 2.5-fold higher than that obtained in FB B and FB C (induced by IPTG and Gal) (Table 2).

Fig. 5 Mass spectrometric analysis of a 24-h reaction catalyzed by 6-OST-1-purified enzymes coming from fermentor runs. Control (without any enzyme addition), FB A, FB B, and FB C are fermentor runs A, B, and C, respectively. Substrate *N*-sulfoheparosan decasaccharide, shows an *m/z* 694.08 ([M-3H]³⁻), corresponding to its molecular weight of 2,085.2886 and product, containing a single *O*-sulfo group shows a *m/z* 720.74 ([M-3H]³⁻), corresponding to a molecular weight of 2,165.2454



Discussion

The efficient expression of recombinant biosynthetic enzymes is critical for the production of sufficient bioengineered heparin for preclinical evaluation. Evaluation of our current process (Wang et al. 2011c; Bhaskar et al. 2012) suggests that from 10 to 100 g of each of the four biosynthetic enzymes will be required to produce a kilogram of bioengineered heparin. Current production of sulfotransferases from *E. coli* grown in shake flasks afford <10 mg l⁻¹ of these enzymes, suggesting the need to move to fed batch fermentors that afford high cell densities.

This study demonstrates that recombinant 6-OST-1 can be prepared in high density fed batch fermentation. The first critical issue that needed to be addressed was the development a semi-defined medium. Glycerol and soya peptone media allows for high cell densities in fed batch fermentations (Restaino et al. 2011). Next, we examined the choice and concentration of inducer. The use of coupled IPTG-Gal inducers resulted in excellent production of 6-OST-1. Gal is partially taken up from the medium and might be utilized as a secondary carbon source. Despite the post-induction decrease in Gal, high levels of 6-OST-1 were obtained in the dual inducer fermentations.

The activity of the recombinant 6-OST-1 was assessed using a newly developed assay involving the direct observation of substrate and product using mass spectrometry.

The specific activity of the 6-OST-1 afforded from three fed batch fermentations ranged from 24 to 82 pmolmin⁻¹mg⁻¹. These activities compare favorably to those previously reported other sulfotransferases (Paul et al. 2012).

In conclusion, the production of 6-OST-1 has been increased by 50-fold along with a 20-fold increase in cell density. These improvements can be credited to the use of a semi-defined medium and a dual inducer strategy. Our cost analysis suggests that a volumetric productivity of 1 gL⁻¹ would be required for these enzymes to transform the process into a feasible one. In this work, we have been able to achieve a productivity level of 455 mgL⁻¹. Future work will be aimed at optimizing the media, induction, and fermentation conditions to maximize the production of 6-OST-1.

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