



Published in final edited form as:

Biotechnol Bioeng. 2010 December 15; 107(6): 964–973. doi:10.1002/bit.22898.

***E. coli* K5 fermentation and the Preparation of Heparosan, a Bioengineered Heparin Precursor**

Zhenyu Wang¹, Mellisa Ly², Fuming Zhang³, Weihong Zhong⁴, Amy Suen², Anne Marie Hickey³, Jonathan S. Dordick^{1,3}, and Robert J. Linhardt^{1,2,3}

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

⁴ Department of Biotechnology, College of Biological & Environmental Engineering, Zhejiang University of Technology, Hangzhou, 310032, PR China

Abstract

Heparosan is an acidic polysaccharide natural product, which serves as the critical precursor in heparin biosynthesis and in the chemoenzymatic synthesis of bioengineered heparin. Heparosan is also the capsular polysaccharide of *Escherichia coli* K5 strain. The current study was focused on the examination of the fermentation of *E. coli* K5 with the goal of producing heparosan in high yield and volumetric productivity. The structure and molecular weight properties of this bacterial heparosan were determined using polyacrylamide gel electrophoresis and Fourier transform-mass spectrometry. Fermentation of *E. coli* K5 in a defined medium using exponential fed batch glucose addition with oxygen enrichment afforded heparosan at 15 g/L having a number average molecular weight of 58,000 Da and a weight average molecular weight of 84,000 Da.

Keywords

fermentation; *E. coli*; capsule; polysaccharide; heparosan; heparin

Introduction

Heparosan is biosynthesized as a polysaccharide capsule in bacteria including *Escherichia coli* K5 and *Pasteurella multocida* (DeAngelis and White 2002; Lindahl et al. 1998; Vann et al. 1981a). The heparosan polysaccharide is comprised of a [\rightarrow 4] β -D-glucuronic acid (GlcA) (1 \rightarrow 4) *N*-acetyl- α -D-glucosamine (GlcNAc) (1 \rightarrow)_n repeating disaccharide unit (Figure 1A). In eukaryotes, heparosan is a precursor in the biosynthesis of heparin and heparan sulfate, biologically important molecules that are involved in blood anticoagulation, viral and bacterial infection and entry, angiogenesis, inflammation, cancer and development. (Linhardt and Toida, 2004; Linhardt 2003) .

Heparin is a uniquely important polysaccharide as it is one of the oldest drugs in use today in widespread clinical prevention of blood coagulation (Linhardt 2003). Isolated by

extraction from porcine intestines (Liu et al., 2009) heparin's use was associated in 2008 with a life-threatening rapid onset, acute side effect (Kishimoto et al. 2008). These adverse events were caused by an oversulfated chondroitin sulfate (OSCS) contaminant found in certain lots of heparin (Guerrini et al. 2008). Laboratory-scale studies have shown that heparosan with a weight average molecular weight (M_w) > 10,000, obtained from *E. coli* K5 strain (Vann et al. 1981a), can be enzymatically converted to an anticoagulant polysaccharide similar to heparin (Lindahl et al. 2005; Zhang et al. 2008). The preparation of a bioengineered heparin from a microbially produced heparosan offers a potentially safer alternative for animal-sourced heparin (Lindahl et al., 2005; Zhang et al., 2008; Laremore et al., 2009) and is the primary focus of this work.

The K5 heparosan polysaccharide, comprising the *E. coli* capsule layer acts as molecular camouflage (Manzoni et al. 1993; Vann et al. 1981b), because of its structural similarity to heparosan, the biosynthetic precursor of heparin and heparan sulfate. Thus, K5 heparosan shows low immunogenicity in humans (Vann et al. 1981b), and this has led to pathogenicity, including urinary infections (Vann et al. 1981b). The initiation of K5 heparosan synthesis reportedly involves 2-keto-3-deoxyoctulosonic acid (Finke et al. 1991). K5 heparosan is then elongated through the alternate action of the glycotransferases KfiA and KfiC that add GlcNAc and GlcA to the nonreducing end of the growing polysaccharide chain (Hodson et al. 2000). Once synthesized, the heparosan chain is transported onto the cell surface through a pathway consisting of six proteins: KpsC, KpsD, KpsE, KpsM, KpsS and KpsT (Arrecubieta et al. 2001; McNulty et al. 2006; Silver et al. 2001; Whitfield and Roberts 1999). The K5 heparosan chain is believed to be anchored on the cell surface through lipid substitution at the reducing end of the polysaccharide to a phosphatidic acid molecule in the outer membrane of *E. coli* (Jann and Jann 1990; M. Alexander Schmidt 1982). Portions of the heparosan polysaccharide can be shed from *E. coli* K5 through the action of K5 heparosan lyase, an enzyme originating from a bacterial phage that cleaves the heparosan chain through a β -elimination mechanism (Hanfling et al. 1996; Manzoni et al. 1996; Manzoni et al. 2000). The gene encoding K5 lyase is integrated into the *E. coli* K5 DNA (Manzoni et al. 1996) and its expression may be inducible (Legoux et al. 1996; Manzoni et al. 1996; Manzoni et al. 2000). The activity of K5 lyase can affect the amount of heparosan released into the culture medium as well as the structure and molecular weight properties of both the cell associated and released heparosan (Figure 1B). K5 heparosan has been estimated to have a M_w 20,000 (Vann et al. 1981a), and comprised of two major subcomponents with M_w 16,000 and 1,500. The ratio of the two subcomponents corresponds to the overall M_w and is influenced by the activity of the K5 lyase (Manzoni et al. 1996; Manzoni et al. 2000).

Heparosan obtained from *E. coli* K5 offers a promising precursor for preparation of a bioengineered heparin as its backbone structure might be carefully modified using chemoenzymatic steps to generate a product identical to animal-derived heparin (Zhang et al., 2008). The control of heparosan molecular weight properties is, therefore, critical because the biological properties of heparin depend strongly on the polysaccharide chain size, which will depend critically on the chain size of the heparosan precursor.

In the current study, we produce heparosan through fermentation of *E. coli* K5 on different media and characterize its chemical structure and molecular weight properties. The yield of heparosan was enhanced by growth on defined media using an exponential feeding strategy, thereby offering the first step toward a scalable process that might one day lead to the industrial production of heparosan as a bioengineered heparin precursor.

Materials and methods

Materials

A 7-L glass autoclavable bioreactor from Applikon (Schiedam, Netherlands) was used as the fermentor. BioXpert V1.5 software was used to control fermentor operation and collect data. Difco™ LB broth powder was purchased from BD (Franklin Lakes, NJ). Most of the chemicals used for preparing the synthetic media were from Sigma-Aldrich (St Louis, MO). Antifoam 204 was from Sigma-Aldrich (St Louis, MO). Baffled shake flasks are from Corning (Corning, NY). DEAE Sepharose Fast Flow from GE Healthcare (Piscataway, NJ) was used for the purification of heparosan. Vivapure D Mini H spin columns were from Sartorius Stedim Biotech (Aubagne, France). Micro BCA Protein Assay Kit was from Thermo Scientific (Rockford, IL). The enzymes used to digest the heparosan for disaccharide analysis were expressed and purified in our lab (Han et al. 2009). HPLC-MS was performed on an Agilent 1100 instrument (Santa Clara, California). TLC silica gel plates were from EMD (Gibbstown, NJ). The materials for preparing the heparosan MW ladder and determining heparosan M_w were described in detail in another paper (Ly et al., 2010). Select_HA LoLadder, a set of hyaluronan molecular markers, were obtained from Hyalose (Oklahoma City, OK).

***E. coli* K5 growth in 2.8 L shake flasks**—*E. coli* K5 from American Type Culture Collection (ATCC #23506) was stored frozen in 1 mL of M9, LB, glycerol and glucose media, with 25% glycerol. A 250 mL shake flask containing 25 mL of the same media was inoculated with 0.5 mL of defrosted *E. coli*. The culture was harvested in late exponential growth at approximately 1.1 g dry cell weight (DCW)/L for M9 medium culture, 5.4 g DCW/L for glycerol synthetic medium, 5.6 g DCW/L for glucose synthetic medium, and 1.9 g DCW/L for LB medium. *E. coli* K5 culture (300 mL) was next grown in 2.8 L shake flasks inoculated with 5 vol % cells in late exponential growth. The culture was shaken at 220 RPM and 37 °C until 1–4 h after growth reached stationary phase and then harvested for recovery of heparosan. The media used in 2.8 L shake flask fermentation included: **1.** LB medium: Difco™ LB broth, Lennox, 20 g/L. **2.** M9 medium: 2 g/L glucose, 0.12 g/L $MgSO_4$, 0.011 g/L $CaCl_2$, 0.337 g/L thiamine-HCl, 6 g/L Na_2HPO_4 , 3g/L KH_2PO_4 , 0.5 g/L NaCl, 1 g/L NH_4Cl . **3.** Glycerol defined medium: 20 g/L glycerol, 10 to 300 mg/L thiamine, 13.5 g KH_2PO_4 ; 4.0 g $(NH_4)_2HPO_4$, 1.4 g $MgSO_4 \cdot 7H_2O$, 1.7 g citric acid, and 10.0 mL trace metal solution. Trace metal solution consisted of (per L of 5 M HCl) 10.0 g $FeSO_4 \cdot 7H_2O$, 2.0 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.02 g $Na_2B_4O_7 \cdot 10H_2O$ (Wang and Lee 1998). **4.** Glucose defined medium: 20 g/L glucose, 10 to 300 mg/L thiamine, 13.5 g KH_2PO_4 ; 4.0 g $(NH_4)_2HPO_4$, 1.4 g $MgSO_4 \cdot 7H_2O$, 1.7 g citric acid, and 10.0 mL trace metal solution. Trace metal solution consisted of (per L of 5 M HCl) 10.0 g $FeSO_4 \cdot 7H_2O$, 2.0 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.02 g $Na_2B_4O_7 \cdot 10H_2O$ (Wang and Lee 1998).

***E. coli* K5 growth in a 7 L fermentor**—This fermentation consists of a batch growth stage and a fed batch growth stage. The composition of the medium for the batch growth in the fermentation was: 20 g/L glucose, 10 or 300 mg/L thiamine, 13.5 g KH_2PO_4 ; 4.0 g $(NH_4)_2HPO_4$, 1.4 g $MgSO_4 \cdot 7H_2O$, 1.7 g citric acid, and 10.0 mL trace metal solution. Trace metal solution consisted of (per L of 5 M HCl) 10.0 g $FeSO_4 \cdot 7H_2O$, 2.0 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.02 g $Na_2B_4O_7 \cdot 10H_2O$. The feeding solution used in the fed batch stage consisted of (per L): 250–1000 g glucose, 20 g $MgSO_4 \cdot 7H_2O$ and 0.15 or 0.25 g thiamine (Wang and Lee 1998). The pH was kept at a value of ~ 7.0 throughout the fermentation.

The batch growth stage began with the inoculation of seed culture (300 mL of 5.6 g/L DCW) obtained from a shake flask in late exponential growth. The temperature was maintained at $\sim 37^{\circ}\text{C}$, and the pH was maintained at approximately 7 (by adding 29% ammonia solution). Air was sparged into the fermentor to supply oxygen, and the stirrer speed was set to 520 RPM.

The second stage of the fermentation began after glucose in the batch growth medium had been depleted and the dissolved oxygen showed a sharp increase. The feeding solution was then fed exponentially following Eq. 1:

$$M_s(t) = F(t)S_{F0} = \left(\frac{\mu}{Y_{x/s}} + m \right) X(t_0)V(t_0)\exp[\mu(t - t_0)] \quad (1)$$

M_s is mass flow rate of the carbon source (g/h); F is feed flow rate (L/h); S_F is carbon substrate concentration in the feed (g/L); X is cell concentration (g/L dcw); m is specific maintenance coefficient (g/g dcw/h); V is culture volume (L); t_0 is time of feeding start; t is process time; μ is specific growth rate (h^{-1}); and $Y_{x/s}$ is cell yield on carbon substrate (g/g) (Lee 1996). A μ value between 0.10–0.15 h^{-1} was used in this study to allow sufficient cell propagation while avoiding accumulation of toxic side products due to higher growth rates. The “acid pump” attached to the fermentor was used to carry out the glucose feeding function instead of adding real acid, and the base pump was used to add 29% ammonia solution to maintain stable pH and provide the culture with a nitrogen source. A third pump was used to add the Antifoam 204 reagent into the culture when foaming exceeded a set level controlled by the feedback loop through the digital control unit of the fermentor. Glucose-feeding rate was achieved by programming the “acid pump” on-off time for one-minute. An equation of $T_{\text{on}} = 0.72 * \exp[0.0023 * (t - 420)]$ was used between 7 h and 24 h of elapsed fermentation time, where T_{on} is the time the “acid pump” was turned on for one second during the one-minute period. The glucose-feeding rate was decreased after 24 h once oxygen limitation was observed. pH control was achieved manually using a base pump to add 29% ammonium hydroxide solution. The pH was more closely controlled using a customized program written into the BioXpert V1.5 software in a second fermentation (see Supplemental Materials). The stirrer speed and/or airflow rate were increased when dissolved oxygen dropped below 20% air saturation. Pure oxygen was mixed with air to afford sufficient dissolved oxygen after 24 h of fermentation time. Glucose feeding was periodically halted to assure that the cells consumed all the glucose, and that toxic byproducts (such as acetate) did not build up in the medium. Feeding was resumed after a spike of dissolved oxygen was observed, indicating the depletion of glucose and the formation of toxic byproducts (Johnston et al. 2002; Johnston et al. 2003; Korz et al. 1995; Lee 1996). Samples were collected from the fermentor periodically. The samples taken were centrifuged at $12,000 \times g$ for 30 min. to separate the supernatant from the *E. coli* cells.

Determination of heparosan concentration in the fermentation supernatant—

The heparosan concentration in the fermentation supernatant was measured by both carbazole analysis (Bitter and Muir 1962) and NMR analysis (Wang et al. 2009). In the carbazole assay of heparosan, 0.5 mL of fermentation supernatant recovered by centrifugation was applied to a Millipore YM-3 desalting spin column ($1200 \times g$) to remove salts and small molecules. The retentate containing crude heparosan was recovered and lyophilized. The dried crude heparosan was reconstituted with 0.5 mL of distilled water and subjected to the carbazole assay (Bitter and Muir 1962). The concentration of heparosan was calculated from a standard curve prepared using pure heparosan. In the NMR assay, heparosan (1 mL), similarly recovered from culture supernatant, was lyophilized and then dissolved in 400 μL of D_2O containing 71 μg of sodium terephthalate (internal standard)

then transferred to an NMR tube for $^1\text{H-NMR}$ at 600 MHz. The concentration of heparosan was calculated from the ratio of the integrated area of the *N*-acetyl group in heparosan and that of the internal standard (Wang et al. 2009).

Rapid recovery of heparosan samples during fermentation for analysis—

Heparosan was rapidly recovered from fermentation supernatant and partially purified using a Vivapure D Mini H spin column. Supernatant (1 mL), recovered from cells by centrifugation ($12,000 \times g$), was mixed with 1 mL of buffer A (50 mM sodium chloride, 20 mM sodium acetate, pH 4). The mixture was then adjusted to pH 4 and loaded onto a pre-equilibrated Vivapure D Mini H column. The column was then washed with buffer A and eluted with buffer B (1 M sodium chloride, 20 mM sodium acetate, pH 4). Eluted samples were then precipitated with 3 volumes of ethanol left overnight at -20°C (in an explosion-proof freezer) and the resulting precipitate washed with 75% ethanol, dissolved in water and lyophilized. The purity of the recovered samples was then examined by $^1\text{H-NMR}$.

Purification of heparosan—

Heparosan was recovered at the completion of fermentation in either a shake flask or the 7-L fermentor by centrifuging the culture at $12,000 \times g$ for 30 min. The supernatant obtained was adjusted to pH 4 by adding glacial acetic acid and then filtered through a Pyrex Buchner funnel with a fritted disc (pore size 40–60 μm). DEAE-Sephacrose fast flow resin was packed into a column of the appropriate size (< 20 mg of heparosan / mL of swelled resin). The column was first equilibrated with 50 mM sodium chloride in 20 mM sodium acetate buffer at pH 4. The fermentation supernatant was then loaded onto the column and the column was washed with 3-volumes of 50 mM sodium chloride in 20 mM sodium acetate buffer at pH 4. Heparosan was then eluted from the column with 1 M sodium chloride in 20 mM sodium acetate buffer at pH 4. The heparosan that eluted from the column was precipitated by adding 3-volumes of ethanol and storing this solution overnight at -20°C in an explosion-proof freezer. The precipitate was recovered by centrifuging at $12,000 g$ for 30 min. The pellet was washed with 75% ethanol, centrifuged again and the pellet obtained was either lyophilized for storage or used directly for the bleaching step. Heparosan could also be purified from the cell pellet by addition of 0.02% SDS followed by vigorous stirring, centrifugation and DEAE chromatography.

In the bleaching step, heparosan was first dissolved in 1 M sodium chloride at a concentration of ~ 15 g/L. The pH of the solution was adjusted to 9.5 with 1 M NaOH and hydrogen peroxide (30%) was added to obtain a final concentration of 1.5%. The mixture was incubated overnight at room temperature, after which the heparosan was precipitated by adding 3-volumes ethanol and left overnight at -20°C (in an explosion-proof freezer). The resulting pellet was washed with 75% ethanol, dissolved in water and dried.

Disaccharide analysis of heparosan—

Heparosan (10 mg/mL) was dissolved in 200 mM sodium phosphate buffer at pH 7 and treated with 1 mU each of heparin lyase 1, 2 and 3 at 30°C overnight. The resulting disaccharide product was subjected to high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-mass spectrometry (MS) on an Agilent Ion trap instrument and the disaccharide composition was determined. LC-MS analysis was performed on an LC-MS system (LC/MSD trap MS; Agilent, Santa Clara, CA). Solutions A and B for high-pressure liquid chromatography were 15% and 65% acetonitrile, respectively, containing the same concentration of 37.5 mM NH_4HCO_3 and 11.25 mM tributylamine. The separation was performed on a C-18 column (Agilent) using solution A for 20 min, followed by a linear gradient from 20 to 45 min of 0–50% solution B. The column effluent entered the source of the electrospray ionization mass spectrometry (ESI-MS), for continuous detection by MS. The electrospray interface was set in negative ionization mode with skimmer potential of 400 V, capillary exit of 240.0 V, and a source of temperature of 325°C , to obtain maximum abundance of the ions in a full-scan spectrum

(150–1,500 Da, 10 full scans/s). Nitrogen was used as a drying (5 liters/min) and nebulizing gas (20 psi) (Bhattacharyya et al., 2010).

NMR analysis of heparosan— ^1H -NMR and ^{13}C -NMR were conducted on a Bruker 600 MHz NMR spectrometer. The heparosan samples were prepared at a concentration of 2 mg/mL in D_2O (99.99+ atom %), freeze-dried to remove exchangeable protons and re-dissolved in D_2O and transferred to standard 5 mm NMR tubes. Acquisition of spectra was carried out using TOPSPIN 2.0 software. All the spectra were acquired at the temperature of 298 K.

DNA and protein content assay—The DNA content in the final heparosan product was determined by measuring the UV absorbance of 0.1 mg/mL heparosan solution at 260 nm and 320 nm. The DNA concentration was calculated as concentration ($\mu\text{g}/\text{mL}$) = $(A_{260} \text{ reading} - A_{320} \text{ reading}) \times \text{dilution factor} \times 50 \mu\text{g}/\text{mL}$. Protein content was assayed using the Micro BCA Protein

Assay Kit following manufacturer's instructions—Analysis of the molecular weight of heparosan A ladder of heparosan standards of known molecular masses, was prepared from bleached K5 heparosan polysaccharide by continuous preparative polyacrylamide electrophoresis using a Mini Prep Cell (Biorad, Hercules, CA) apparatus (Ly et al., 2010). Heparosan fractions were characterized by Fourier transform-mass spectrometry (FT-MS) and remixed to prepare a ladder of molecular weight standards for the determination of the molecular weight properties of heparosan (Ly et al., 2010). Molecular markers of hyaluronan (HA), which is also linear polysaccharide with the same charge density as N-acetylheparosan, were used as standards for the upper range of samples.

Both HA and heparosan molecular markers were used as a set of standards for the molecular weight analysis of the heparosans prepared in different culture media and at different fermentation time points. A gradient polyacrylamide gel (4–15%) of dimensions 0.75 mm \times 6.8 cm \times 8.6 cm was used in heparosan molecular weight analyses. Heparosan samples (25 μg) were loaded onto gels and were then subjected to electrophoresis (200 V for 20 min) and stained with Alcian blue for 1 h, and then destained with 25% ethanol/10% acetic acid (Ly et al., 2010). The gels were scanned and these digital images were analyzed using the computer software UN-SCANIT. A plot of image density as a function of migration distance was acquired. From these data, the molecular weight properties of the heparosan samples were characterized from the standard curve obtained.

Results

E. coli K5 fermentation in shake flasks for the preparation of heparosan

E. coli K5 was grown in shake flasks until 1–4 h after stationary phase was reached. The fermentation was extended beyond stationary phase to accumulate the maximum amount of heparosan in the medium, consistent with reports that heparosan appearance in the medium lags cell growth (Manzoni et al. 1993). The yield of heparosan from the flask cultures ranged from 70 – 500 mg/L. The purity of the heparosan recovered from flask cultures was in all cases > 85% as estimated by NMR (Wang et al. 2009; Zhang et al. 2008). The heparosan recovered from synthetic media, including M9 medium, glucose medium and glycerol medium, showed higher purity levels (> 95%) than heparosan recovered from LB medium, which showed additional peaks in the NMR, consistent with ~ 85% purity (Figure 2). Further analysis of the LB medium derived heparosan by polyacrylamide gel electrophoresis (PAGE) showed a low M_w band, corresponding to a medium component

that co-purifies with heparosan. This impurity could be removed using a 3K molecular weight cut-off (MWCO) spin column (Figure 2A).

The disaccharide composition of heparosan was determined by HPLC-MS (not shown) following the complete digestion with heparin lyase 1, 2 and 3, and showed the presence of a single disaccharide m/z 378.2. This disaccharide corresponds to Δ UA(1 \rightarrow 4)- α -D-GlcNAc, is consistent with the uniform repeating structure of heparosan, \rightarrow 4)- β -D-GlcA(1 \rightarrow 4)- α -D-GlcNAc(1 \rightarrow) (Vann et al. 1981a). To confirm this structure $^{13}\text{C}'$ and $^{15}\text{N}'$ labeled heparosan was prepared by culturing the *E. coli* K5 in M9 medium containing uniformly labeled ^{13}C -glucose and ^{15}N -ammonium chloride. The structure of the recovered uniformly labeled ^{13}C -, ^{15}N -heparosan was confirmed by complete digestion with heparin lyase 1, 2 and 3 followed by HPLC-MS, which showed a single disaccharide m/z 392.9. The 15 amu difference between m/z 378.2 and 392.9 is consistent with the full ^{13}C and ^{15}N isotopic enrichment of heparosan and confirms the heparosan repeating structure.

***E. coli* K5 fermentation in a 7 L fermentor**

Since the production of *E. coli* polysaccharide capsule is likely to be growth associated, a strategy was devised to maximize the cell density to increase the yield of heparosan. Oxygen and glucose are the two nutrients likely to limit biomass production in an aerobic fermentation on glucose (Lee 1996; Shiloach and Fass 2005). The presence of excess glucose, however, can decrease cell density through the formation of toxic products, such as acetate (Lee 1996; Shiloach and Fass 2005). Thus, the strategy used was to feed glucose at a rate supporting exponential growth once the glucose present in the initial stage of the fermentation had been depleted.

The feeding rate was exponentially increased to keep up with the exponential growth of *E. coli*. Several pauses were made in the feeding stage to ensure that there was no buildup of glucose in the medium, which could result in acetate accumulation and inhibit *E. coli* growth. The feeding was resumed only after an increase in dissolved oxygen was observed confirming the depletion of glucose (Figure 3). In the late stages of the fermentation (after 22 h), dissolved oxygen became limiting. Increased agitation and the introduction of pure oxygen were used to maximize the dissolved oxygen concentration in the culture. Despite these efforts the dissolved oxygen levels still remained quite low after 31 h. The glucose-feeding rate was decreased when oxygen became the limiting nutrient. Throughout the fermentation, the pH was maintained between 6 and 8 by adding NH_4OH . After 37.5 h fermentation, a cell density of 85 g DCW/L was reached, and the heparosan concentration in the fermentation supernatant was determined to be 15 g/L. An overall growth rate was calculated to be 0.12 h^{-1} , the overall production rate was 1.2 g/h, and the volumetric production rate was $0.4 \text{ g/L}\cdot\text{h}$. The heparosan purified from the fermentation was of high purity, judging from the ^1H -NMR spectrum (Figure 4A). Additionally, DNA and BCA protein assays indicated that <1% DNA and <2% protein were present in the final heparosan material.

Structural characterization of heparosan

^1H -NMR, ^{13}C -NMR, HPLC-MS and Fourier Transform (FT)-MS (Figure 4) all confirm the general structure of heparosan as \rightarrow 4)- β -D-GlcA(1 \rightarrow 4)- α -D-GlcNAc(1 \rightarrow) (Figure 1A). FT-MS of a heparosan fraction having a degree of polymerization (dp) of 24, obtained using preparative electrophoresis is shown in Figure 4C. This spectrum also indicates the presence of unsaturated uronate Δ UA residue (Figure 1B) at the non-reducing end of some of the polysaccharide chains. The presence of a terminal Δ UA residue is consistent with the action of an K5 heparosan lyase also present in the *E. coli* strain K5 (Hanfling et al. 1996; Manzoni et al. 2000). FT-MS analysis suggests heparosan recovered from fermentation supernatant

contains a mixture of heparosan terminated with Δ UA residues and with GlcA residues. This result is consistent with some of the heparosan in the medium being shed from the cell surface by shear force and some being released into the medium by the heparosan lyase digestion. Heparosan isolated from the supernatant was treated with β -glucuronidase, an exolytic enzyme capable of removing GlcA but not DUA from the non-reducing end of a heparosan chain. The observation of released GlcA using thin layer chromatography (data not shown) confirms that some of the heparosan chains that were shed into the supernatant were terminated with GlcA.

The ^{13}C NMR spectrum of the isotope labeled heparosan from M9 medium (Figure 4B) showed no signal clearly assignable to the Δ UA residue consistent with previous reports (Manzoni et al. 1996). This may result from low levels of K5 lyase on M9 medium or the short culture times used for growth in shake flasks compared to the longer growth times in fermentor, where the action of the K5 heparosan lyase was clearly observed. We performed an additional experiment in the fermentor (see Supplemental Materials) where we used gel based and ultraviolet assays capable of sensitively detecting coliphage lyase (Ly et al., 2010). In this study we were unable to detect lyase activity in the fermentation supernatant; however, we did find a small level of lyase activity in the cell lysate that was constant throughout the fermentation. These data are consistent with the suggestion that coliphage lyase is present in the cytoplasm or periplasm (Linhardt et al. 1986; Hudson et al. 2009).

MW characterization of heparosan

The heparosans recovered from *E. coli* K5 grown in shake flasks, were analyzed by PAGE against a ladder of purified heparosan standards (Figure 5 and Table 1). Heparosan, recovered from supernatant in flask cultures grown on M9 medium, glycerol synthetic medium, LB complex medium showed little difference in M_n or M_w . Heparosan recovered from the supernatant in glucose synthetic medium had the lowest M_n and M_w . Heparosan recovered from the pellet showed higher M_w than heparosan recovered from the fermentation supernatant (data not shown).

Next, samples from the fermentor were taken at various times throughout the fermentation and heparosan was purified by Vivapure D Mini H spin column. The purity of the resulting samples was confirmed to be > 85% from the fermentation supernatants by $^1\text{H-NMR}$. PAGE analysis (Figure 5B) showed an initial increase in heparosan molecular weight followed by no further changes in molecular weight as fermentation time increases (Figure 6). Heparosan polydispersity index ($\text{PDI} = M_w/M_n$) changed little throughout the fermentation.

Discussion

In this study, a high heparosan yield in the fermentation supernatant (15 g/L) was achieved in a fed-batch fermentor culture grown on defined medium containing glucose. This compares favorably to yields recently reported of 10.2 g/L heparosan from the same organism grown on a defined media containing glycerol (Viskov et al., 2008). Volumetric production rate was also increased compared to this previous report. Furthermore, glucose, as a less expensive carbon source than glycerol, makes this fermentation process more economical (Viskov et al., 2008). Capsule synthesis, while lagging behind cell growth, is nonetheless growth-associated at 37°C (Cieslewicz and Vimr 1996; Rowe et al. 2000). Therefore, our primary goal was to maximize the volumetric productivity of the *E. coli* K5 fermentation via control of the carbon source and its concentration, and the dissolved oxygen concentrating, which becomes the limiting nutrient at high cell densities. Exponential feeding of glucose was used to support cell growth, while keeping toxic byproducts low. This was achieved by controlling the feeding rate and checking the substrate level with feeding pauses. Oxygen became the limiting nutrient for cell growth in

the late stages of the fermentation. Oxygen was used to supplement air sparging to increase cell density. However, the use of pure oxygen in large-scale heparosan might be problematic from a process economics point of view. The fed-batch fermentation process developed at the 3-L scale might serve as the prototype for larger lab-scale fermentation, ultimately leading to industrial heparosan production. Process scale-up studies are currently underway in our lab moving from 3 L to 750 L working volumes, which will provide the basis for scale-up to the ~100,000 L working volumes required for large-scale (100 metric ton) heparosan production to supply as the starting material for bioengineered heparin synthesis. The possibility of using a faster glucose feeding rate in the fermentation and higher oxygen levels in the fermentor is being investigated to increase the production rate the heparosan.

The use of defined media containing glucose as the carbon source has both lowered the fermentation cost and reduced media complexity, making the purification process easier. Heparosan purified from complex LB medium was less pure due to complex media components and required additional purification steps than did heparosan purified from glucose-containing defined media.

Heparosan, isolated from the culture supernatant arises from shedding into the medium during the fermentation probably through the combined actions of the K5 lyase and shear force. An increase of heparosan shedding into the media increases the yield of heparosan in the supernatant, lowering the cost of the down-stream purification process. Several ways to enhance capsule shedding including genetic manipulation are under investigation in our lab.

The presence of the K5 heparosan lyase complicates our effort to prepare a heparosan with ideal structural and molecular weight properties (M_n , M_w , and PDI) as the starting material for bioengineered heparin. First, the β -eliminative cleavage of the heparosan chain, catalyzed by the lyase results in an unnatural saccharide residue, Δ UA, at the non-reducing end of the chain. Because Δ UA is not present in the animal-sourced heparin chain, additional chemical, enzymatic or metabolic engineering steps will be required to remove this unnatural sugar residue so that the bioengineered heparin will closely resemble the natural animal sourced heparin. This close structural resemblance is necessary as it will allow bioengineered heparin to be treated as a generic heparin and be subject to an abbreviated new drug application (ANDA)-based Food and Drug Administration (FDA)-approval process. Second, the action of lyase during the fermentation time course increases the polydispersity index of heparosan. This, in turn, complicates our effort to control the molecular weight properties of heparosan. This is a critical issue as the molecular weight properties of bioengineered heparin are inexorably linked to those of heparosan. In the presence of the lyase it should still be possible to prepare heparosan with the desired molecular weight properties by carefully controlling the fermentation conditions and the fermentation processing time. Careful process control can be used to balance chain polymerization with K5 lyase action. The presence of K5 lyase contributes to the desirable release of the heparosan capsule into the medium, increasing the heparosan yield. Thus, deletion of the K5 lyase gene, while resulting in a more natural heparosan without a double bond at the non-reducing end of the chain and a narrower polydispersity, might decrease the yield of heparosan fermentation. Furthermore, the cell-association of K5 lyase might offer an approach to for the fine control heparosan molecular weight, necessary for the production of a bioengineered heparin having ideal molecular weight properties. Future studies will be required to achieve an appropriate balance between ideal heparosan precursor and high heparosan yield for the commercial production of a generic bioengineered heparin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to the National Institutes of Health for supporting this research with grants GM38060 and HL096972.

References

- Alexander M, Schmidt KJ. Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extraintestinal infections. *FEMS Microbiol Lett.* 1982; 14:69–74.
- Arrecubieta C, Hammarton TC, Barrett B, Chareonsudjai S, Hodson N, Rainey D, Roberts IS. The transport of group 2 capsular polysaccharides across the periplasmic space in *Escherichia coli*. Roles for the KpsE and KpsD proteins. *J Biol Chem.* 2001; 276(6):4245–50. [PubMed: 11078739]
- Bhattacharyya S, Solakyildirim K, Zhang Z, Linhardt RJ, Tobacman JK. Cell-bound IL-8 increases in bronchial epithelial cells after arylsulfatase B silencing due to sequestration with chondroitin-4-sulfate. *Am J Respir Cell and Molec Biol.* 2010; 42:51–61. [PubMed: 19346317]
- Bitter T, Muir HM. A Modified Uronic Acid Carbazole Reaction. *Analytical Biochemistry.* 1962; 4(4): 330. [PubMed: 13971270]
- Chuang YJ, Swanson R, Raja SM, Olson ST. Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin. *J Biol Chem.* 2001; 276(18): 14961–71. [PubMed: 11278930]
- Cieslewicz M, Vimr E. Thermoregulation of kpsF, the first region 1 gene in the kps locus for polysialic acid biosynthesis in *Escherichia coli* K1. *J Bacteriol.* 1996; 178(11):3212–20. [PubMed: 8655501]
- DeAngelis PL, White CL. Identification and molecular cloning of a heparosan synthase from *Pasteurella multocida* type D. *J Biol Chem.* 2002; 277(9):7209–13. [PubMed: 11756462]
- Finke A, Bronner D, Nikolaev AV, Jann B, Jann K. Biosynthesis of the *Escherichia coli* K5 polysaccharide, a representative of group II capsular polysaccharides: polymerization in vitro and characterization of the product. *J Bacteriol.* 1991; 173(13):4088–94. [PubMed: 1829455]
- Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, Capila I, Lansing JC, Guglieri S, Fraser B, et al. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nature Biotechnology.* 2008; 26(6):669–675.
- Guerrini M, Zhang Z, Shriver Z, Masuko S, Langer R, Casu B, Linhardt RJ, Torri G, Sasisekharan R. Orthogonal analytical approaches to detect potential contaminants in heparin. *Proc Nat Acad Sci USA.* 2009; 106:16956–16961. [PubMed: 19805108]
- Han YH, Garron ML, Kim HY, Kim WS, Zhang Z, Ryu KS, Shaya D, Xiao Z, Cheong C, Kim YS, et al. Structural snapshots of heparin depolymerization by heparin lyase I. *J Biol Chem.* 2009; 284(49):34019–27. [PubMed: 19801541]
- Hanfing P, Shashkov AS, Jann B, Jann K. Analysis of the enzymatic cleavage (beta elimination) of the capsular K5 polysaccharide of *Escherichia coli* by the KS-specific coliphage: A reexamination. *Journal of Bacteriology.* 1996; 178(15):4747–4750. [PubMed: 8755913]
- Hodson N, Griffiths G, Cook N, Pourhossein M, Gottfridson E, Lind T, Lidholt K, Roberts IS. Identification that KfiA, a protein essential for the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide, is an alpha -UDP-GlcNAc glycosyltransferase. The formation of a membrane-associated K5 biosynthetic complex requires KfiA, KfiB, and KfiC. *J Biol Chem.* 2000; 275(35): 27311–5. [PubMed: 10859322]
- Hudson T, Goldrick M, Roberts IS. The *Escherichia coli* K5 capsule is not synthesized in a protected compartment within the cytoplasm. *J Bacteriol.* 2009; 191(5):1716–8. [PubMed: 19074385]
- Jann B, Jann K. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. *Curr Top Microbiol Immunol.* 1990; 150:19–42. [PubMed: 2404688]
- Johnston W, Cord-Ruwisch R, Cooney MJ. Industrial control of recombinant *E. coli* fed-batch culture: new perspectives on traditional controlled variables. *Bioprocess Biosyst Eng.* 2002; 25(2):111–20. [PubMed: 14505011]

- Johnston WA, Stewart M, Lee P, Cooney MJ. Tracking the acetate threshold using DO-transient control during medium and high cell density cultivation of recombinant *Escherichia coli* in complex media. *Biotechnol Bioeng.* 2003; 84(3):314–23. [PubMed: 12968285]
- Kishimoto TK, Viswanathan K, Ganguly T, Elankumaran S, Smith S, Pelzer K, Lansing JC, Sriranganathan N, Zhao GL, Galcheva-Gargova Z, et al. Contaminated heparin associated with adverse clinical events and activation of the contact system. *New England Journal of Medicine.* 2008; 358(23):2457–2467. [PubMed: 18434646]
- Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J Biotechnol.* 1995; 39(1):59–65. [PubMed: 7766011]
- Laremore TN, Zhang F, Dordick JS, Liu J, Linhardt RJ. Recent progress and applications in glycosaminoglycan and heparin research. *Curr Opin Chem Biol.* 2009; 13:633–640. [PubMed: 19781979]
- Lee SY. High cell-density culture of *Escherichia coli*. *Trends Biotechnol.* 1996; 14(3):98–105. [PubMed: 8867291]
- Legoux R, Lelong P, Jourde C, Feuillerat C, Capdevielle J, Sure V, Ferran E, Kaghad M, Delpech B, Shire D, et al. N-acetyl-heparosan lyase of *Escherichia coli* K5: gene cloning and expression. *J Bacteriol.* 1996; 178(24):7260–7264. [PubMed: 8955411]
- Lindahl U, Kusche-Gullberg M, Kjellen L. Regulated diversity of heparan sulfate. *J Biol Chem.* 1998; 273(39):24979–24982. [PubMed: 9737951]
- Lindahl U, Li JP, Kusche-Gullberg M, Salmivirta M, Alaranta S, Veromaa T, Emeis J, Roberts I, Taylor C, Oreste P, et al. Generation of "Neoheparin" from *E coli* K5 capsular polysaccharide. *J Med Chem.* 2005; 48(2):349–352. [PubMed: 15658847]
- Linhardt RJ, Galliher PM, Cooney CL. Polysaccharide lyases. *Appl Biochem Biotechnol.* 1986; 12(2):135–76. [PubMed: 3521491]
- Linhardt RJ. Heparin: structure and activity. *J Med Chem.* 2003; 46:2551–2554. [PubMed: 12801218]
- Linhardt RJ, Toida T. Role of glycosaminoglycans in cellular communication. *Acc Chem Res.* 2004; 37:431–438. [PubMed: 15260505]
- Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. *Nat Prod Rep.* 2009; 26:313–321. [PubMed: 19240943]
- Ly M, Wang Z, Laremore T, Zhang F, Zhong W, Pu D, Zagorevski D, Dordick JS, Linhardt RJ. Analysis of *E. coli* K5 capsular polysaccharide heparosan. *Anal Bioanal Chem.* 201010.1007/s00216-010-3679-7
- Manzoni M, Bergomi S, Cavazzoni V. Extracellular K5 Polysaccharide of *Escherichia-Coli* - Production and Characterization. *Journal of Bioactive and Compatible Polymers.* 1993; 8(3):251–257.
- Manzoni M, Bergomi S, Cavazzoni V. Production of K5 polysaccharides of different molecular weight by *Escherichia coli*. *Journal of Bioactive and Compatible Polymers.* 1996; 11(4):301–311.
- Manzoni M, Bergomi S, Rollini M. Influence of the culture conditions on extracellular lyase activity related to K5 polysaccharide. *Biotechnology Letters.* 2000; 22(1):81–85.
- McNulty C, Thompson J, Barrett B, Lord L, Andersen C, Roberts IS. The cell surface expression of group 2 capsular polysaccharides in *Escherichia coli*: the role of KpsD, RhsA and a multi-protein complex at the pole of the cell. *Mol Microbiol.* 2006; 59(3):907–22. [PubMed: 16420360]
- Rowe S, Hodson N, Griffiths G, Roberts IS. Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. *J Bacteriol.* 2000; 182(10):2741–5. [PubMed: 10781541]
- Shiloach J, Fass R. Growing *E. coli* to high cell density--a historical perspective on method development. *Biotechnol Adv.* 2005; 23(5):345–57. [PubMed: 15899573]
- Silver RP, Prior K, Nsahlai C, Wright LF. ABC transporters and the export of capsular polysaccharides from gram-negative bacteria. *Res Microbiol.* 2001; 152(3–4):357–64. [PubMed: 11421283]
- Vann WF, Schmidt MA, Jann B, Jann K. The Structure of the Capsular Polysaccharide (K5 Antigen) of Urinary-Tract-Infective *Escherichia-Coli* 010-K5-H4 - a Polymer Similar to Desulfo-Heparin. *European Journal of Biochemistry.* 1981a; 116(2):359–364. [PubMed: 7018909]

- Vann WF, Schmidt MA, Jann B, Jann K. The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* O10:K5:H4. A polymer similar to desulfo-heparin. *Eur J Biochem.* 1981b; 116(2):359–64. [PubMed: 7018909]
- Viskov, C.; Lux, F.; Gervier, R.; Colas, G. Method for producing K5 polysaccharide. US Patent. 2008/0032349. 2008.
- Wang FL, Lee SY. High cell density culture of metabolically engineered *Escherichia coli* for the production of poly(3-hydroxybutyrate) in a defined medium. *Biotechnology and Bioengineering.* 1998; 58(2–3):325–328. [PubMed: 10191411]
- Wang Z, Zhang Z, McCallum SA, Linhardt RJ. Nuclear magnetic resonance quantification for monitoring heparosan K5 capsular polysaccharide production. *Anal Biochem.* 2009
- Whitfield C, Roberts IS. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol.* 1999; 31(5):1307–19. [PubMed: 10200953]
- Yoshida K, Miyauchi S, Kikuchi H, Tawada A, Tokuyasu K. Analysis of unsaturated disaccharides from glycosaminoglycuronan by high-performance liquid chromatography. *Anal Biochem.* 1989; 177(2):327–32. [PubMed: 2499215]
- Zhang ZQ, McCallum SA, Xie J, Nieto L, Corzana F, Jimenez-Barbero J, Chen M, Liu J, Linhardt RJ. Solution structures of chemoenzymatically synthesized heparin and its precursors. *Journal of the American Chemical Society.* 2008; 130(39):12998–13007. [PubMed: 18767845]

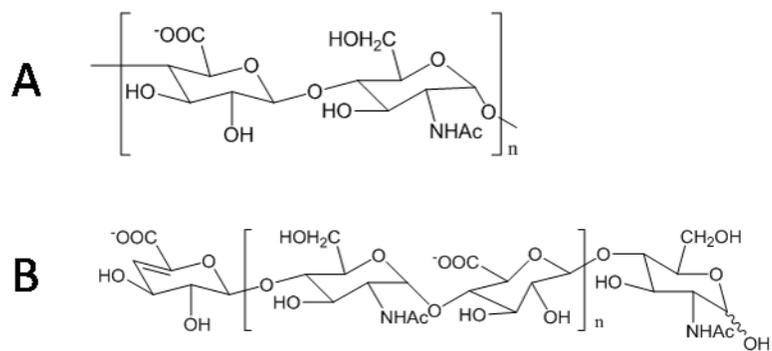


Figure 1. Structure of heparosan. A. repeating disaccharide unit of heparosan B. structure of heparosan chain resulting from the action of heparosan K5 lyase.

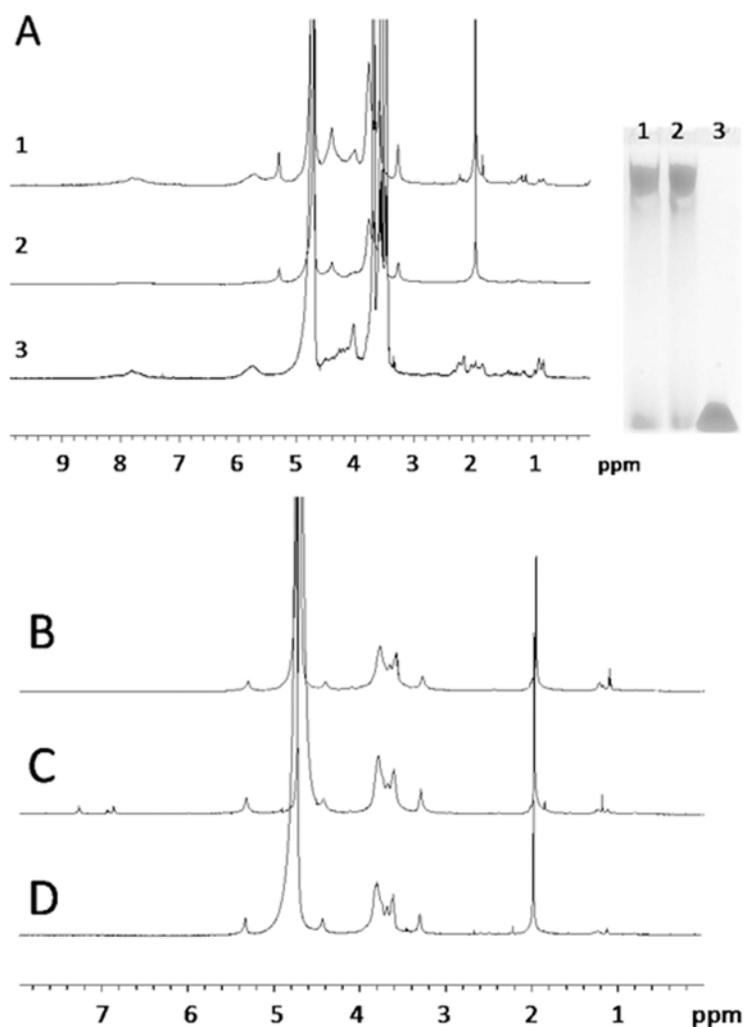


Figure 2. ^1H -NMR spectra (600 MHz) of heparosan prepared in shake flask culture. A. stack plot of heparosan recovered from: 1. LB medium, 2. LB medium having $\text{MW}_{\text{Avg}} > 3,000$, 3. LB medium having $\text{MW}_{\text{Avg}} < 3,000$, and inset showing PAGE analysis of samples 1, 2 and 3. B. heparosan recovered from M9 medium. C. heparosan recovered from glycerol defined medium. D. heparosan recovered from glucose defined medium.

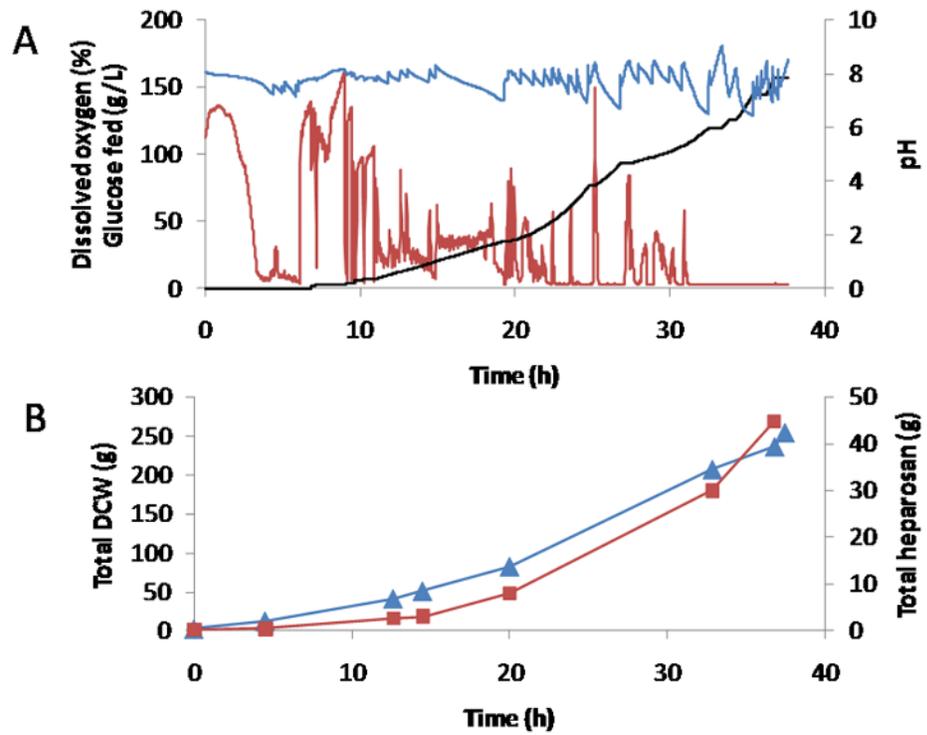


Figure 3. Production of heparosan in a 7 L fermentor. Panel A shows the glucose feed curve (black), the pH curve (blue) and the dissolved oxygen (% DO) curve (red) as a function of fermentation time (h). Panel B shows the cell growth curve (total DCW g, \blacktriangle) and heparosan production (g, \blacksquare) as a function of fermentation time (h).

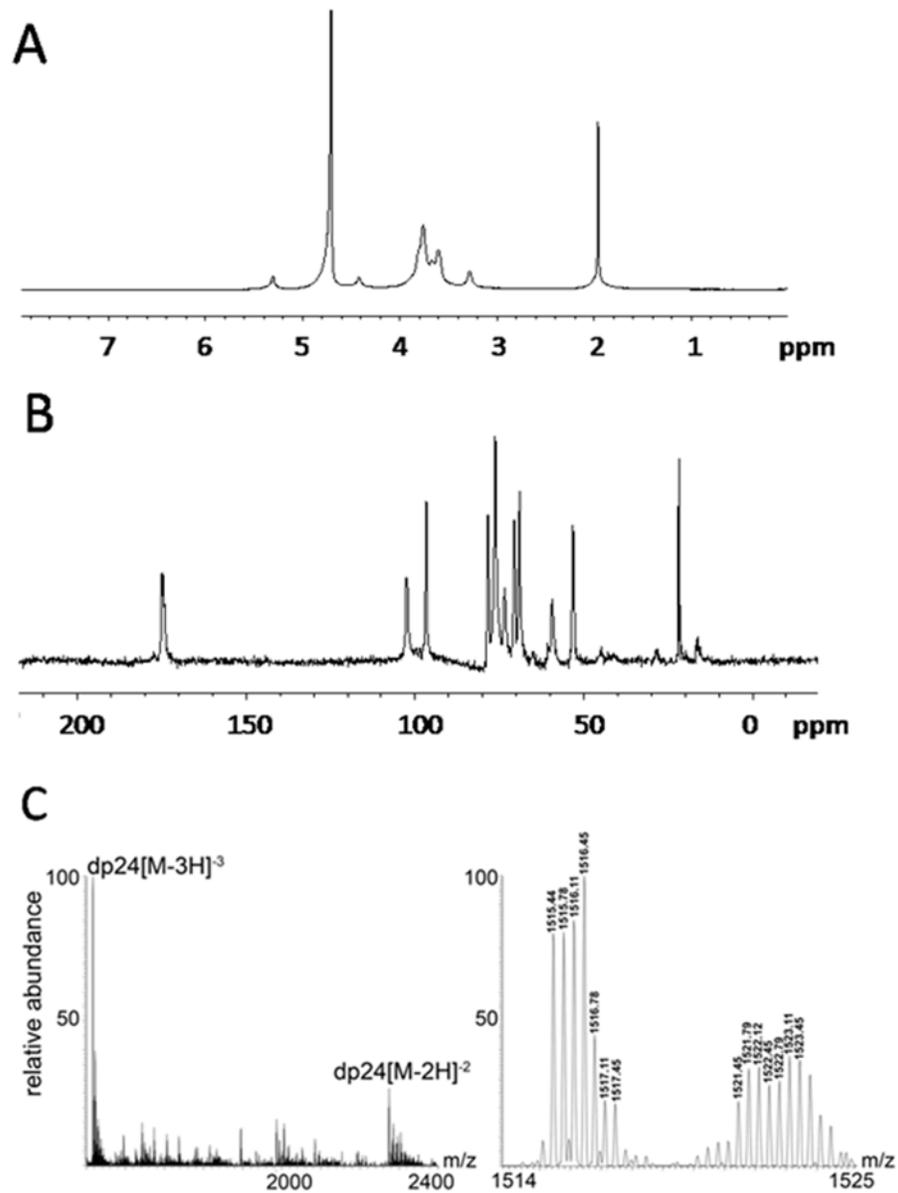


Figure 4. Characterization of heparosan purified from the supernatant of a 7 L fermentation. A. ^1H -NMR (600 MHz) of heparosan. B. ^{13}C -NMR of heparosan prepared on M9 medium containing ^{13}C glucose and ^{15}N ammonia sulfate. C. FT-MS of a heparosan chain of average molecular weight 4551.81 (degree of polymerization =24) purified by preparative PAGE (Ly et al., 2010).

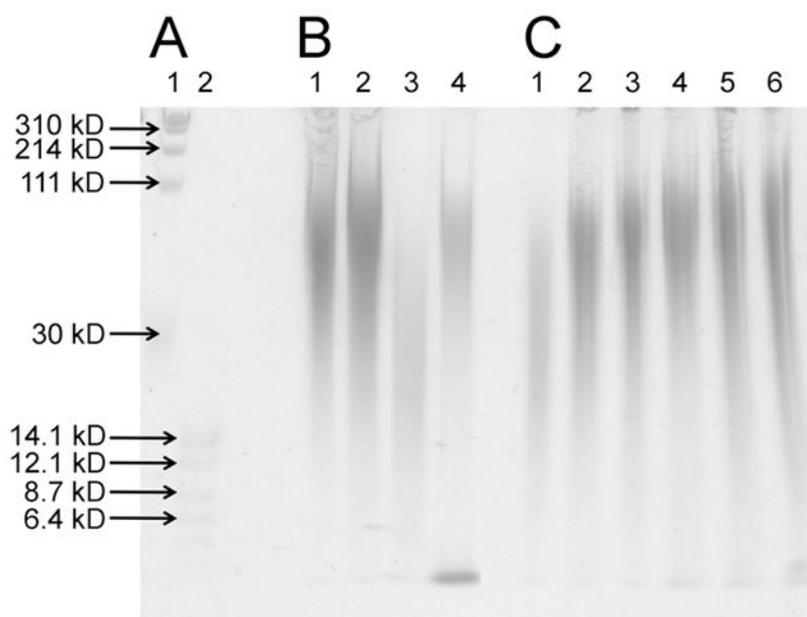


Figure 5. PAGE used for MW analysis of heparosans with Alcian blue stain. A. molecular standards spanning the range of the gradient gel. Lanes contain: 1. HA molecular markers (30 kD-310 kD), 2. heparosan molecular markers (6.4 kD-14.1 kD). B. heparosans prepared in shake flasks in different media. Lanes contain: 1. heparosan from M9 medium, 2. heparosan from glycerol synthetic medium, 3. heparosan from glucose synthetic medium, and 4. heparosan from LB medium. C. heparosans prepared in a 7 L fermentor sampled at different times. Lanes contain: 1–6. heparosan sampled from the fermentor at 4.5 h; 12.6 h; 14.5 h; 20 h; 32.9 h; and 37.6 h after the start of the fermentation.

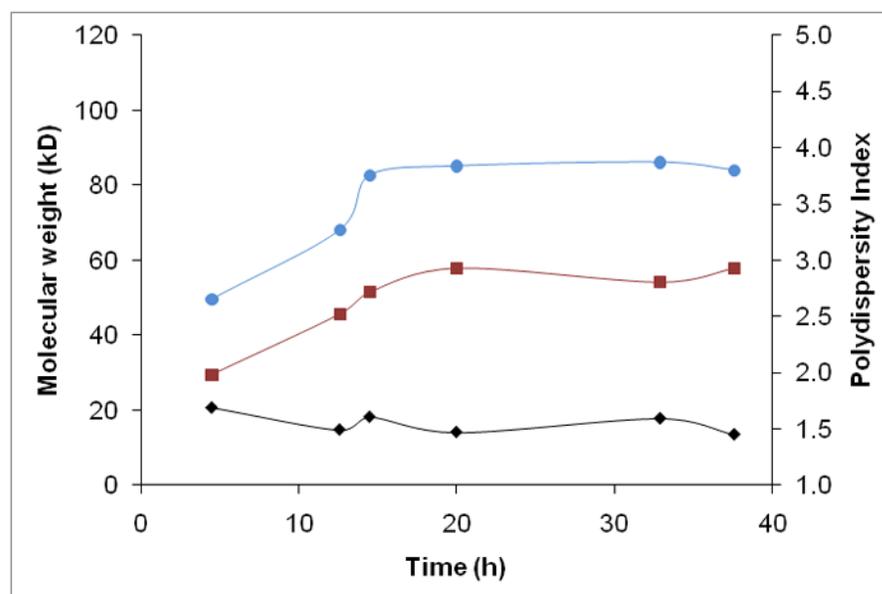


Figure 6. Time course of molecular weight properties of heparosan produced in 7 L fermentor. Shown are the trends of M_N (■), M_W (●) and PDI (◆).

Table 1

Number average molecular weight (M_N), weight average molecular weight (M_W) and polydispersity index (PDI) of heparosan recovered from different media.

Medium	M_N	M_W	PDI
M9	54,000	82,000	1.5
Glycerol	50,000	79,000	1.6
Glucose	25,000	44,000	1.8
LB	54,000	68,000	1.3