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Production of chondroitin in metabolically engineered *E. coli*

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ABSTRACT

Chondroitin sulfates, widely used in the treatment of arthritis, are glycosaminoglycans extracted from food animal tissues. As part of our ongoing efforts to separate the food chain from the drug chain, we are examining the possibility of using metabolic engineering to produce chondroitin sulfate in *Escherichia coli*. Chondroitin is a valuable precursor in the synthesis of chondroitin sulfate. This study proposes a safer and more feasible approach to metabolically engineer chondroitin production by expressing genes from the pathogenic *E. coli* K4 strain, which natively produces a capsular polysaccharide that shares the similar structure with chondroitin, into the non-pathogenic *E. coli* BL21 Star™ (DE3) strain. The ePathBrick vectors, allowing for multiple gene addition and expression regulatory signal control, are used for metabolic balancing needed to obtain the maximum potential yield. The resulting engineered strain produced chondroitin, as demonstrated by ^{1}H NMR and disaccharide analysis, relying on chondrotinase treatment followed by liquid chromatography–mass spectrometry. The highest yield from shake flask experiment was 213 mg/L and further increased to 2.4 g/L in dissolved oxygen-stat fed batch bioreactor.

1. Introduction

Chondroitin sulfate (CS) is an important homocopolymeric glycosaminoglycan (GAG), consisting a repeating disaccharide unit as its backbone, β1→3 or β1→4 linked D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc), and substituted at various positions with *O*-sulfo groups (Fig. 1). It is widely found in the extracellular matrix of animal connective tissue and works together with other fibrous protein to sustain the cell structure and provide porous pathway for nutrients and oxygen diffusion (Schiraldi et al., 2010). The major application for CS is as anti-inflammatory drug for treating osteoarthritis and has been recommended by European League against Rheumatism as a symptomatic slow acting drug for knee and hand osteoarthritis treatment (Michel et al., 2002; Volpi, 2009). CS inhibits cartilage degradative enzymes and its chondroprotective properties may enhance the biosynthesis of connective tissue and increase the synovial fluid viscosity at disease sites (Belcher et al., 1997; McCarty et al., 2000). Clinical studies suggest that CS treatment relieves pain and stiffness caused by arthritis with minor side effects such as increasing intestinal gases and stool softening (Schiraldi et al., 2010). While CS shows low oral bioavailability, it can exhibit its anti-inflammatory activity by acting within the intestine to systemically release interleukins (Sakai et al., 2006; Volpi, 2003). Currently CS is produced from animal tissues including bovine trachea, pig nasal septa, chicken keel and shark fins. Potential risk of interspecies viral and prion transmission, such as bovine spongiform encephalopathy and epizootic aphtha is of growing concern (Schiraldi et al., 2010). There is also concern that massive fishing for shark may result in species extinction (Schiraldi et al., 2010). With the aging of the world population, the demand for CS in treating osteoarthritis is dramatically increasing. Developing a safe and reliable biotechnological process to replace the traditional animal source of this product is the goal of the current research.

Bacterial capsules are protective coatings on the outside surface of bacteria that act as molecular camouflage against the recognition of host immune response (Avci and Kasper, 2010; Cress et al., 2014). The capsular polysaccharide (CPS) of *Escherichia coli* K4 shares a common non-sulfated repeating disaccharide unit (→4) GlcAβ(1→3)GalNAcβ(1→), but contains an extra β-linked fructose at the 3-position of glucuronic acid (Rodriguez et al., 1988). The similarity of this CPS to chondroitin provides a possible approach utilizing microbial fermentation to produce CS.

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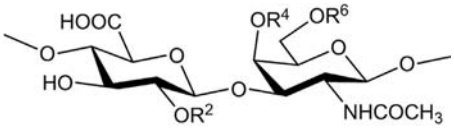
The CPS of K4 belongs to the group II K antigen (Whitfield, 2006). The gene cluster responsible for CPS biosynthesis is organized into three regions, where region I and region III are conserved for all group II K antigens. Region II contains 7 genes of K4, namely *kfoA* to *kfoG*, as well as an insertion *IS2* gene, with a total length of 14 Kb; these genes encode for the enzymes that direct the synthesis and assemble of a chondroitin-like polysaccharide (Rodriguez et al., 1988). The function of KfoB, KfoD and KfoG still remains unknown. Many studies suggested that they might not directly be involved in the capsular polysaccharide production (Doherty, 2011; Krahulec et al., 2005; Ninomiya et al., 2002). The *kfoA* gene encodes the enzyme uridine diphosphate (UDP)-GlcNAc 4-epimerase, responsible for the epimerization of UDP-GlcNAc to UDP-GalNAc. The *kfoF* gene encodes the enzyme UDP-glucose dehydrogenase, involved in the redox reaction where NAD^+ is being reduced to NADH and UDP-glucose is oxidized to UDP-GlcA. The *kfoC* gene encodes a chondroitin polymerase that operates in a dual-action mode to transfer both GlcA and GalNAc residues to the non-reducing end of an oligosaccharide/polysaccharide acceptor (DeAngelis et al., 2002; Ninomiya et al., 2002). The *kfoE* gene is believed to encode a fructosyl-transferase, responsible for the addition of a fructose group on the 3-position of GlcA (Antonio et al., 2011; Liu et al., 2014). This reaction may occur during or after the biosynthesis of the chondroitin backbone is complete (Antonio et al., 2011). A detailed biosynthetic pathway of *E. coli* K4 CPS is illustrated in Fig. 2.



CS-A: GlcAβ1-3GalNAc(4S)
CS-B: IdoA(2S) β1-3GalNAc(4S)
CS-C: GlcAβ1-3GalNAc(6S)
CS-D: GlcA(2S) β1-3GalNAc(6S)
CS-E: GlcAβ1-3GalNAc(4,6S)
CS-F: GlcA(α1-3Fuc)β1-3GalNAc(4S)
CS-O: GlcAβ1-3GalNAc

Fig. 1. Structure of CS.

Several studies related to the optimization of *E. coli* K4 growth have been performed to improve the production and yield of K4 CPS (Cimini et al., 2010b; Manzoni et al., 1996; Restaino et al., 2011; Zoppetti and Oreste, 2004). More recently, increasing attention has been focused on genetic modification and the preparation of recombinant strains to produce CPS. Several strategies, including modifying genes and protein directly related to capsule biosynthesis were proved to significantly improve CPS yield (Cimini et al., 2010a; Doherty, 2011; Zanfardino et al., 2010). Other approaches regarding transcription factors that regulate transcription of the CPS biosynthesis operon are also believed to have a great impact (Cimini et al., 2013; Wu et al., 2013).

Although production levels for *E. coli* K4 CPS are quite high, *E. coli* K4 is a pathogenic bacterium due to the presence of virulence factors (Cress et al., 2014). For example *E. coli* K4 can cause urinary tract infections (Johnson, 1991; Moxon and Kroll, 1990; Wiles et al., 2008). Therefore, a safer, non-pathogenic alternative host, such as *E. coli* BL21 Star™ (DE3), widely used for protein and peptide expression, is

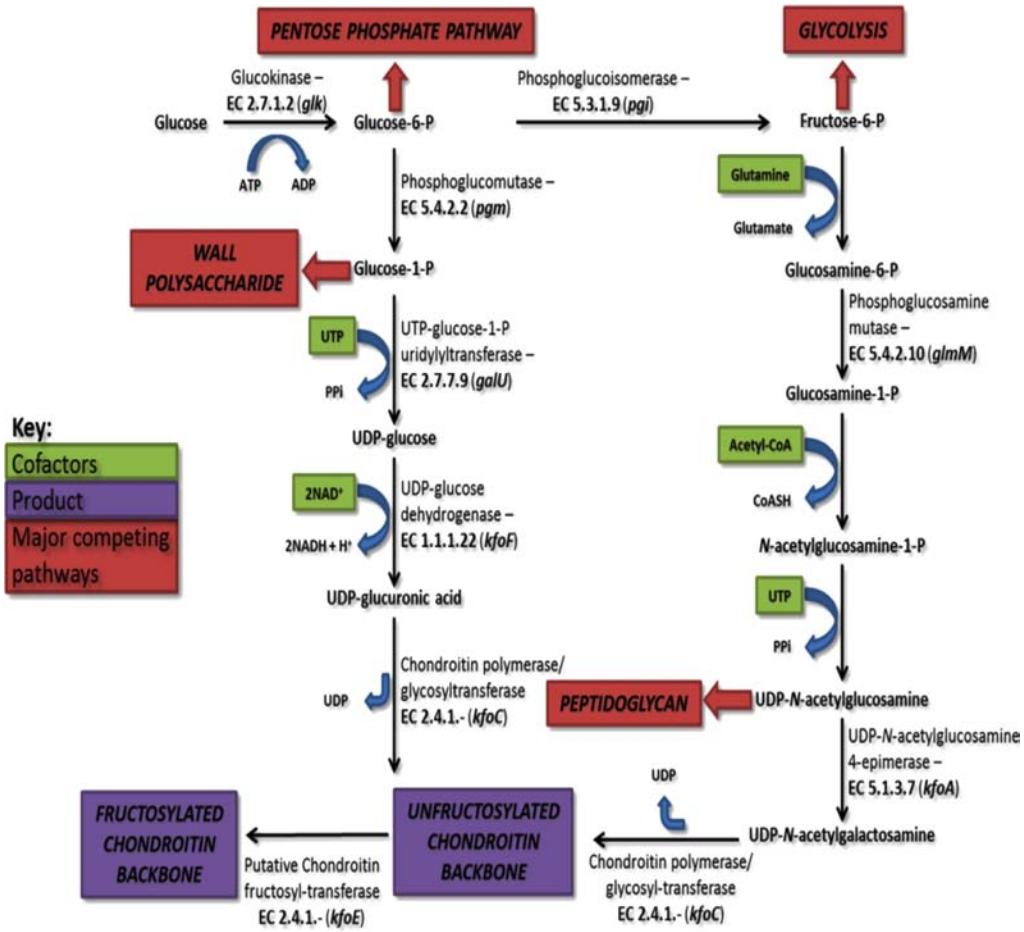


Fig. 2. Metabolic pathway for the synthesis of *E. coli* K4 CPS.

preferable. The *E. coli* BL21 Star™ (DE3) strain has proven to be a suitable host for metabolic engineering in expressing non-native genes for producing a multitude of natural products, including flavonoids, fatty acids and heparosan (Bhan et al., 2013; Xu et al., 2013; Zhang et al., 2012). *E. coli* BL21 Star™ (DE3) is derived from the *E. coli* B strain, which was originally encapsulated; however, insertion of the *IS1* element inactivated its native capsular biosynthesis. The gene cluster involved in transporting mechanism in regions I and III is still active, (Andreishcheva and Vann, 2006) therefore, we hypothesized that we should be able to reestablish CPS biosynthesis in BL21. In the current study, three genes directly related to the biosynthesis of chondroitin were expressed in *E. coli* BL21 Star™ (DE3) using the ePathBrick system, consisting of 5 compatible plasmids with different antibiotics and replication origins. The four isocaudamer pairs (SpeI, XbaI, NheI and AvrII) on these vectors allow for multiple gene addition with various regulatory control signals (Xu et al., 2012). In addition, these promoters allow the construction of gene clusters with different gene configurations, something that has been shown to have significant effect on overall production and yields (Xu et al., 2012). In the present study we used the highest copy number vector and constructed the *E. coli* K4 chondroitin biosynthetic pathway in a pseudo-operon structure for putative maximum yield. While a non-specific carbazole assay has been widely used for quantification of chondroitin production in previous studies (Bitter and Muir, 1962; Wu et al., 2013; Zhang et al., 2012), this assay has been shown to be grossly inaccurate as several media components and bacterial contaminants interfere with it. The current study relies on a more structurally specific and accurate analysis method for quantification of chondroitin. Overall, this is the first successful demonstration of the high-titer production of chondroitin in a non-pathogenic *E. coli* strain, something that opens the way for the future production of other chondroitin-derived polysaccharides of pharmaceutical and commercial importance.

2. Materials and methods

2.1. Media

Luria-Bertani (LB) medium with or without ampicillin (80 μg/ml) was used for the cell growth and transformation screening. Super optimal broth with catabolite repression (SOC) was used for cell recovery after heat shock or electroporation during the transformation experiments. Rich defined medium developed from modified protocols (Cirino et al., 2006; Neidhardt et al., 1974) was used for all the shake flask fermentations (3.5 g/l KH_2PO_4, 5.0 g/l K_2HPO_4, 3.5 g/l $(NH4)_2HPO_4$, 2 g/l casamino acids, 100 ml of 10 × MOPS Mix, 1 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$, 1 ml of 0.5 g/l Thiamine HCL, supplemented with 20 g/l glucose. 10 × MOPS Mix consisted of 83.7 g/l MOPS, 7.2 g/l Tricine, 28 mg/l $FeSO_4 \cdot 7H_2O$, 29.2 g/l NaCl, 5.1 g/l NH_4Cl, 1.1 g/l $MgCl_2$, 0.5 g/l K_2SO_4, 0.2 ml Micronutrient Stock. Micronutrient Stock consisted of 0.2 g/l $(NH_4)_6Mo_7O_{24}$, 1.2 g/l H_3BO_3, 0.1 g/l $CuSO_4$, 0.8 g/L $MnCl_2$, 0.1 g/l $ZnSO_4$. *E. coli* K4 serotype 05:K4 (L):H4 was purchased from American Type Culture Collection (ATCC 23502). *E. coli* BL21 Star™ (DE3) was used as the production strain with expression of ePathBrick plasmid containing *kfoA*, *kfoC* and *kfoF* (Table 1). All the nutrients and chemicals for medium preparation were from Sigma Chemical Co. (St. Louis, MO).

2.2. Plasmid construction

Genomic DNA of *E. coli* K4 was isolated by genomic DNA extraction kit (Invitrogen). Each target gene *kfoA*, *kfoC* and *kfoF* was amplified out by polymerase chain reaction (PCR) using Accuzyme® mix (BIOLINE) according to the manufacturer's instructions. The primers used are listed in supplement material and were designed based on the complete genome sequence of K4 (Cress et al., 2013), and cloned into the pETM6 vector, the highest copy number plasmid in the ePathBrick system. Accordingly, all three genes were assembled into pseudo-operon configuration containing a T7 promoter for each target gene and a single terminator at the end of the last gene (Xu et al., 2012) (Fig. 3). Plasmid DNA was prepared by E.Z.N.A plasmid mini kit (OMEGA) and digested DNA fragments were recovered from agarose gel (Bio-Rad) by E.Z.N.A. gel extraction kit (OMEGA). FastDigest Restriction endonuclease and Rapid DNA ligation kit were purchased from Thermo. Both *kfoA* and *kfoC* contain a SpeI restriction site inside the gene. Silent mutations (*kfoA* 15Thr (t-a) and *kfoC*, 432Leu (c-t); 573Thr (t-a)) were introduced by site direct mutagenesis using QuikChange® Site-Directed Mutagenesis Kit (Agilent). The codon of each mutation was optimized to the most codon usage in *E. coli* K4 (Doherty, 2011) and verified by both double endonuclease digestion and DNA sequencing (Genewiz). Plasmid construction followed standard techniques and ePathBrick platform protocol. Finally the plasmids were transformed into *E. coli* BL21 Star™ (DE3) by electroporation using Bio-Rad Gene Pulser Xcell™ transformation system (2 mm cuvettes, 2.5 kV, 25 μF and 200 Ω). Cells were recovered in super optimal broth with catabolite repression (SOC) medium for 50 min and plated on LB plate, supplemented with 80-μg/ml of ampicillin for screening.

Table 1
List of plasmids and strains used in this study.

Strain/plasmid	Description	Source/reference
Strain		
DH5α	General cloning host	Invitrogen
E. coli K4	Wild type Serotype O5:K4(L):H4	ATCC
BL21Star™ (DE3)	ompT hsdT hsdS (rBmB) gal(DE3)	Novagen
Plasmid		
pETM6	T7 promoter, ColE1 ori. Amp^R	Xu's paper
pETM6_kfoA	pETM6 carrying gene *kfoA* from *E. coli* K4	This study
pETM6_kfoC	pETM6 carrying gene *kfoC* from *E. coli* K4	This study
pETM6_kfoF	pETM6 carrying gene *kfoF* from *E. coli* K4	This study
pETM6_OACF	pETM6 carrying genes in the order of *kfoA*, *kfoC* and *kfoF* from *E. coli* K4 in operon configuration	This study
pETM6_PACF	pETM6 carrying genes in the order of *kfoA*, *kfoC* and *kfoF* from *E. coli* K4 in pseudo-operon configuration	This study
pETM6_MACF	pETM6 carrying genes in the order of *kfoA*, *kfoC* and *kfoF* from *E. coli* K4 in monocistronic configuration	This study
pETM6_PAFC	pETM6 carrying genes in the order of *kfoA*, *kfoF* and *kfoC* from *E. coli* K4 in pseudo-operon configuration	This study
pETM6_PCAF	pETM6 carrying genes in the order of *kfoC*, *kfoA* and *kfoF* from *E. coli* K4 in pseudo-operon configuration	This study
pETM6_PCFA	pETM6 carrying genes in the order of *kfoC*, *kfoF* and *kfoA* from *E. coli* K4 in pseudo-operon configuration	This study
pETM6_PFAC	pETM6 carrying genes in the order of *kfoF*, *kfoA* and *kfoC* from *E. coli* K4 in pseudo-operon configuration	This study
pETM6_PFCA	pETM6 carrying genes in the order of *kfoF*, *kfoC* and *kfoA* from *E. coli* K4 in pseudo-operon configuration	This study

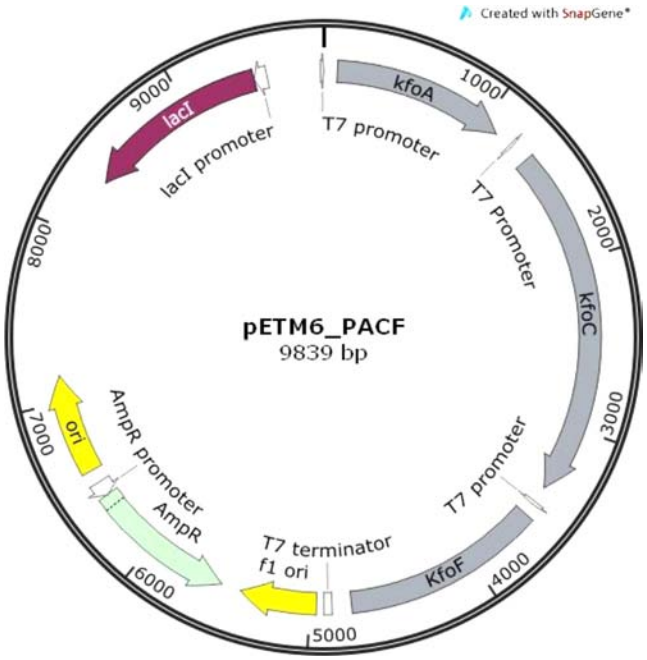


Fig. 3. The ePathbrick construct containing the three genes encoding for chondroitin biosynthesis.

2.3. Shake flask experiments

Shake flask experiments were carried out to evaluate the effect of gene order in pseudo-operon structure on chondroitin production. For each construct in *E. coli* BL21, cells from 15% glycerol stock were streaked on an agar plate containing 80-μg/ml of ampicillin and grown overnight. Two colonies from each plate were picked for duplicate sample analysis and pre-cultures were grown overnight at 37 °C. The samples were then diluted to 25 ml at optical density (OD) ~0.05 and transferred to a 250 ml Erlenmeyer flask and incubated at 37 °C with shaking at 220 rpm. Gene expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD ~1.0 and the cultures were left to grow under the same conditions for an additional 18 to 24 h. The medium for shake flask experiments is presented in the medium section and was supplemented with 80-μg/ml of ampicillin.

2.4. Fermentor experiments

The fed-batch (DO) start control mode fermentation was performed in a 2 L bioreactor (BIOFLO110, New Brunswick Scientific Co., Edison, NJ) with defined medium described in Section 2.1. Strain BL21 Star™ (DE3) with pETM6_PCAF, which gave the optimal yield in shake flask experiments, was used to test the chondroitin production potential. Seed culture (100 ml) was prepared by picking a single colony from the plate and incubated at 37 °C overnight. Then the 100 ml seed culture was inoculated into 1 l of defined media (pH 6.8 and appropriate antibiotics). The bioreactor was first operated at batch mode for 8.5 h and was switched to the fed-batch mode by feeding 40% glucose (w/w) at a constant flow rate of 0.13 ml/min. pH was maintained at 6.8 throughout the fermentation by adjusting with 5 N NH_4OH. The aeration rate speed was maintained at 2.0 volume per volume-minute (vvm). Agitation varied from 500 to 1000 ppm. Recombinant gene expression was initiated by inducing with 0.5 mM IPTG and switching the temperature from 37 °C to 33 °C at 6 h cultivation in batch mode (OD ~6). Samples were taken every 1–4 h for monitoring residual glucose level, cell density and chondroitin production.

2.5. Chondroitin purification

Purification of capsular polysaccharide was carried as follows. First, cell pellet was re-suspended in water and autoclaved in the liquid cycle for 15 min. The supernatant was collected and centrifuged to remove insoluble material. Both autoclaved supernatant from the cell pellet and cell culture supernatant were precipitated with 80 vol% cold ethanol and stored in an explosion-proof refrigerator at −20 °C overnight allowing the recovery of both intracellular and extracellular chondroitin. After precipitation, pellet was collected and re-suspended in digestion buffer (100 mM Tris, pH 7.5, 50 mM $MgCl_2$, 10 mM $CaCl_2$). DNAse (1 mg/l, Sigma) was added and the sample incubated at 37 °C for 1 h. Protease K (2.5 mg/ml, Sigma) was then added and the sample was incubated at 56 °C for 2 h. A second precipitation from 80% cold ethanol was then carried out and the dry pellet was collected, re-dissolved in water (~1 ml) and filtered through a 10 KDa spin column to remove residual small peptides and salt. The retentate was lyophilized for future NMR analysis.

2.6. NMR analysis

The purified CPS from both supernatant and cell pellet were analyzed by one-dimensional ^1H nuclear magnetic resonance (NMR) (Fu et al., 2013). All NMR experiments were performed on a Bruker Advance II 600 MHz spectrometer (Bruker BioSpin, Billerica, MA) with Topsin 2.1.6 software (Bruker). Samples were each dissolved in 0.5 ml D_2O (99.996%, Sigma Chemical Company) and freeze-dried repeatedly to remove the exchangeable protons. The samples were re-dissolved in 0.4 ml D_2O and transferred to NMR microtubes (outside diameter, 5 mm, Norell (Norell, Landisville, NJ)). The conditions for one-dimensional ^1H NMR spectra were as follows: wobble sweep width of 12.3 kHz, acquisition time of 2.66 s, and relaxation delay of 8.00 s. Temperature was 298 K (Fu et al., 2013). De-fructosylated CPS from *E. coli* K4 (chondroitin, a generous gift from Dr. Nicola Volpi of the University of Modena, Italy) was used as an NMR standard to confirm assignments.

2.7. Quantification of chondroitin using HPLC–MS

The use of colorimetric assays, such as carbazole (Bitter and Muir, 1962) for quantification of GAGs derived from bacteria fermentation is limited by interference from medium and cellular debris. Disaccharide analysis using HPLC–MS offers a structurally specific assay for chondroitin quantification (Yang et al., 2012).

Structure characterization of the polysaccharide produced from engineered BL21 Star™ (DE3) strain by HPLC–MS has previously been described (Yang et al., 2012). Since the composition of disaccharide in the samples was much simpler while the standard assay that separates different chondroitin sulfate took a significant amount time (80 min/sample), a shorter assay (16 min/sample) described below was developed accordingly to increase the overall efficiency of analysis.

Complete depolymerization of chondroitin was performed using chondroitinase ABC. The purified CPS from both supernatant and cell pellet were dissolved in 100 μL digestion buffer (50 mM ammonium acetate, 2 mM calcium acetate, pH 7.5). Chondroitinase ABC (20 mU in 5 μl of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4)) was added and incubated at 35 °C for 10 h to depolymerize chondroitin. The digested solution was lyophilized. The freeze-dried samples containing chondroitin disaccharides (~5 μg) or chondroitin disaccharide standard (5 μg, Iduron, UK) was added to 10 μl of a 0.1 M AMAC solution in acetic acid (AcOH)/dimethyl sulfoxide (DMSO) (3:17, v/v) and mixed by vortexing for 5 min. Next, 10 μl of 1 M $NaBH_3CN$ was added in the reaction mixture and incubated at 45 °C for 4 h. Finally, the AMAC-tagged

disaccharide was diluted to different concentrations (0.5–50 ng) using 50% (v/v) aqueous DMSO and LC–MS analysis was performed.

Liquid chromatography mass spectrometry (LC–MS) analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with a 6300 ion-trap and a binary pump. The column used was a Poroshell 120C18 column (3.0×30 mm^2, 2.7 μm, Agilent, USA) at 55 °C. Eluent A was 80 mM ammonium acetate solution and eluent B was methanol. Solution A and 20% solution B was flown (200 μl/min) through the column for 4 min followed by linear gradients 40% solution B from 4 to 60 min. The column effluent entered the electrospray ionization–MS source for continuous detection by MS. The electrospray interface 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, to obtain the maximum abundance of the ions in a full-scan spectrum (300–1200 Da). Nitrogen (8 l/min, 40 psi) was used as a drying and nebulizing gas.

3. Results

3.1. Plasmid construction

PCR was used to amplify chondroitin biosynthetic genes from *E. coli* K4 genomic DNA (*kfoA* 1.02 kbp, *kfoC* 2.06 kbp and *kfoF* 1.17 kbp). Recombinant plasmids pETM6-*kfoA*, pETM6-*kfoC* and pETM6-*kfoF* were verified by double endonuclease digestion and DNA sequencing. Further sub-cloning for construction of pETM6-PACF was verified by restriction digest (digestion of pETM6-PACF with EcoRI, SmaI and SpeI yield three fragments of 1.57, 2.32 and 5.95 kbp, respectively). The same verification method was used to verify cloning of all other plasmids described in Table 1. After electroporation, *E. coli* BL21 Star™ (DE3) containing the desired plasmid was selected from agar plate with ampicillin and an extra step of double digestion verification was performed to further verify successful cloning.

3.2. Characterization of chondroitin structure with NMR and disaccharide analysis

As seen in the chromatogram of Fig. 4, no peak matches the disaccharide standard of chondroitin (0S) for BL21 Star™ (DE3) expressing pETM6 (negative control) while clear peaks of chondroitin (0S) were observed for sample of BL21 Star™ (DE3) expressing pETM6_PACF. This confirmed the production of chondroitin when the three genes *kfoA*, *kfoC* and *kfoF* were expressed in *E. coli* BL21 Star™ (DE3). In addition, NMR analysis further proved the presence of this polysaccharide.

The chemical structure of chondroitin was determined by one-dimensional ^{1}H NMR. Two samples, negative control (N.C.) consisting of BL21 Star™ (DE3) carrying empty pETM6 vector and recombinant strain BL21 Star™ (DE3) carrying plasmid pETM6-PACF were prepared as previously described. In addition, pure de-fructosylated K4 CPS standard was analyzed for comparison (Fig. 5). In the spectra, none of the anomeric proton signals of GlcA and GalNAc as well as the *N*-acetyl group signal was observed in the negative control sample, which indicates that the BL21 Star™ (DE3) with pETM6 plasmid did not produce any K4 polysaccharide. However, all the proton signals perfectly matched with the standard as shown in the expression strain sample (Fig. 5). There is only a very small amount of impurities observed in the high field region. These NMR results confirmed the capability of engineered BL21 Star™ (DE3) strains to produce identical disaccharide repeat units to the pure chondroitin backbone.

3.3. Shake flask experiment and 2 L fed-batch fermentation

Rich defined medium for the shake flask experiment is generally a complex medium such as LB that contains tryptone, a pancreatic digested casein, which is from an animal derived source and can potentially contain GAGs. In order to avoid interference in chemical structural characterization and quantification, no complex medium was used in the shake flask experiments in this study. *E. coli* K4 wild type was compared with the recombinant *E.*

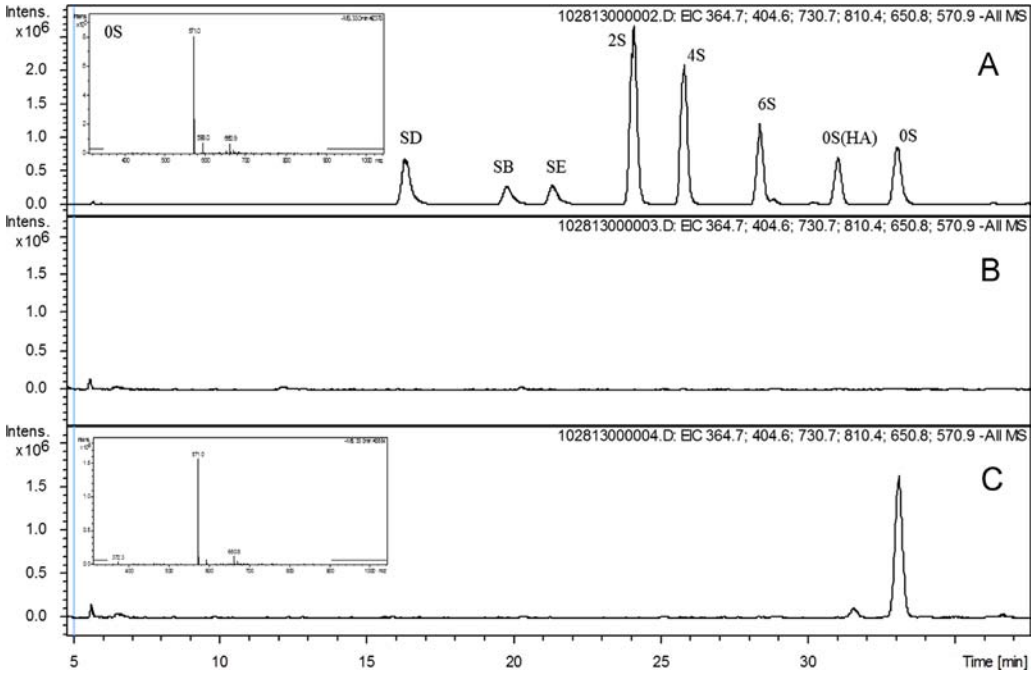


Fig. 4. Disaccharide analysis chromatogram of chondroitin. (A) The standard EIC spetra showing 8 different disaccharide compositions corespondingly to their mass. The mass of 0S(chondroitin) is illustrated in the left with. (B) The sample prepared from fermentation of *E. coli* BL21 Star™ (DE3) with the vector pETM6 as a negative control. No background disaccharide compositions are observed when no biosynthesis genes are expressed. (C) The sample prepared from fermentation of *E. coli* BL21 Star™ (DE3) with the vector pETM6_PACF. The signature peak is observed correlated to the 0 S (chondroitin) in standard suggesting the presence of the product.

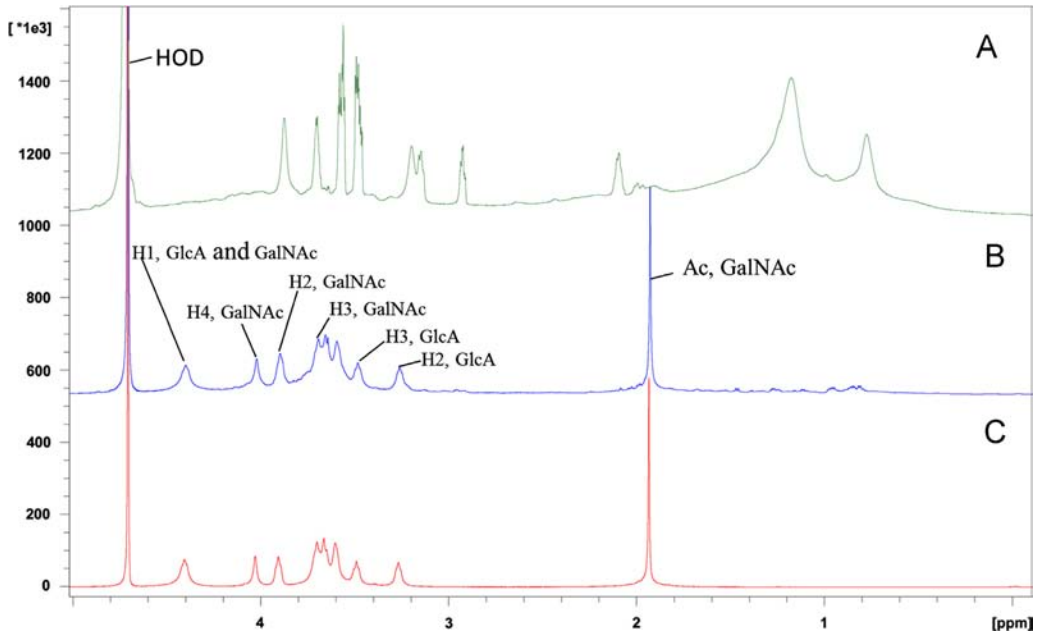


Fig. 5. One-dimensional NMR spectral analysis of produced chondroitin. (A) The sample was from the previously described negative control. No obvious symbolic peaks were observed for chondroitin. (B) The samples were prepared from fermentation of *E. coli* BL21 Star™ (DE3) with the vector pETM6_PACF. The peaks labeled closely matched to the signature pick of chondroitin standard proving that the recombinant chondroitin had identical chemical structure as chondroitin. (C) The standard spectra of chondroitin.

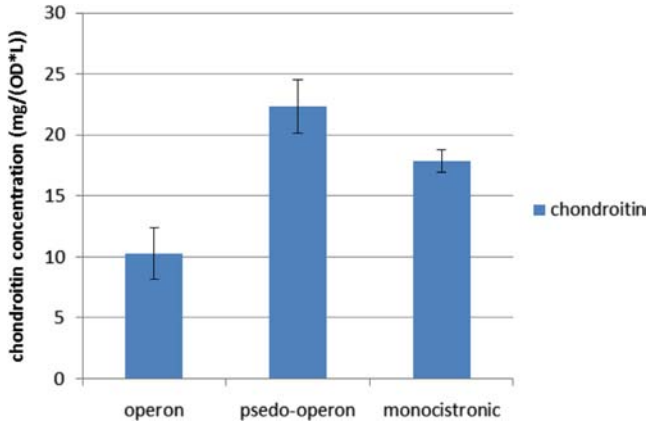


Fig. 6. The production of chondroitin from *E. coli* BL21 Star™ (DE3) with different configurations, operon, pseudo-operon and monocistronic in the gene order of *kfoA*, *kfoC* and *kfoF* was compared. The pseudo-operon structure gave the optimal yield while operon configuration gave the least optimal.

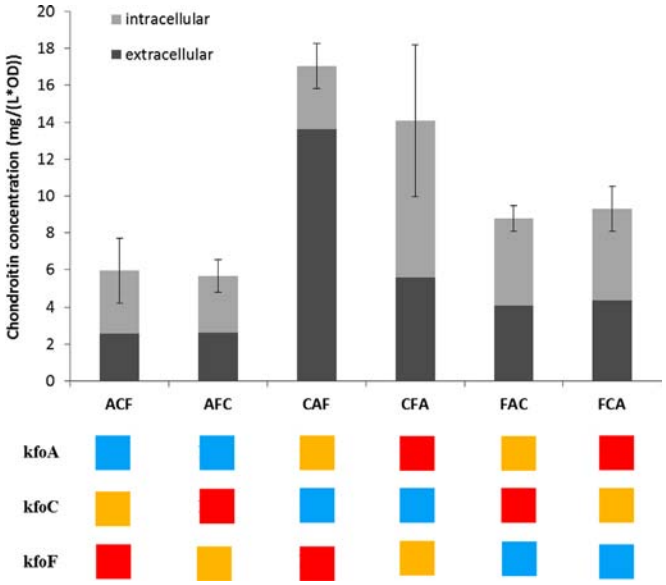


Fig. 7. Chondroitin production in recombinant *E. coli* BL21 Star™ (DE3) as a function of pseudo-operon configuration.

coli BL21 Star™ (DE3) strain (Supplemental material). Based on growth curves, the antibiotics added did not place too much of a burden to the cell growth. The recombinant *E. coli* BL 21 strain grew slightly slower after induction and delayed reaching maximum cell density by 2 h. However, the overall growth and glucose uptake were similar. Three different configurations with the same gene order were first tested and the results are shown in Fig. 6. As expected from previous studies, pseudo-operon structure resulted in maximum yield with a 117% increase compared to the operon structure. The optimal constructs, regarding gene orders in pseudo-operon structure, were further tested and the highest yield of chondroitin is 213 ± 9.87 mg/l with construct of pETM6-PCAF. Detailed production level of different constructs is presented in Fig. 7.

The production of *E. coli* capsular polysaccharide is commonly considered as growth associated. Therefore a strategy used in the past to optimize chondroitin production yield from *E. coli* K4 relied on maximizing cell density. Glucose was fed at a limited flow rate of 0.052 g/min to avoid accumulation of toxic byproduct acetic acid that potentially decreases the cell growth rate. The fermentation was terminated after the optical density reading (OD) started to drop after ~50 h. Glucose level was monitored closely and several pauses were made during the feeding stage to ensure the depletion of glucose. The maximum OD reached ~39.9 after 40 h while the maximum chondroitin production reached 1.9 ± 0.042 g/l after 50 h.

A complete fermentation sample analysis on both supernatant and total chondroitin production is important for better understanding the transport mechanism in BL21 Star™ (DE3).

No chondroitin was detected in either supernatant or cell pellet during the first hour after induction (Fig. 8). After two hours, the concentration ratio of chondroitin from pellet to that from supernatant reached a constant pellet: supernatant ratio of 5:1, suggesting that the transport of polysaccharide to the supernatant is highly restricted. This ratio became larger after 20 h and the product concentration in the supernatant remained relatively low until the fermentation stopped.

4. Discussion

The current microbial approach to prepare chondroitin relies on *E. coli* K4 strain and requires post-fermentation processing to remove fructose residues. This study provides a more direct approach, expressing only the three essential genes involved in the K4 CPS biosynthesis pathway in the non-pathogenic *E. coli* BL21 Star™ (DE3) strain. The highest fermentation yield of chondroitin obtained in the current study was 2.4 g/l, which is comparable with current literature reports of *E. coli* K4 CPS production. Previous studies have focused primarily on overexpression of *kfoC*, a gene that encodes a dual function enzyme that catalyzes both chondroitin polymerization and glycosylation. The overexpression of *kfoC* can be used to direct both GalNAc and GlcA pathways towards CPS production. However, KfoC crystal structure revealed that efficient binding of UDP sugar to its corresponding catalytic site may be another key factor in controlling CPS production (Osawa et al., 2009). One approach, from the aspect of binding strength, suggests that an increase in the affinity for UDP-GalNAc and reduction in the UDP-GlcA interaction would increase K4 CPS production (Zanfardino et al., 2010).

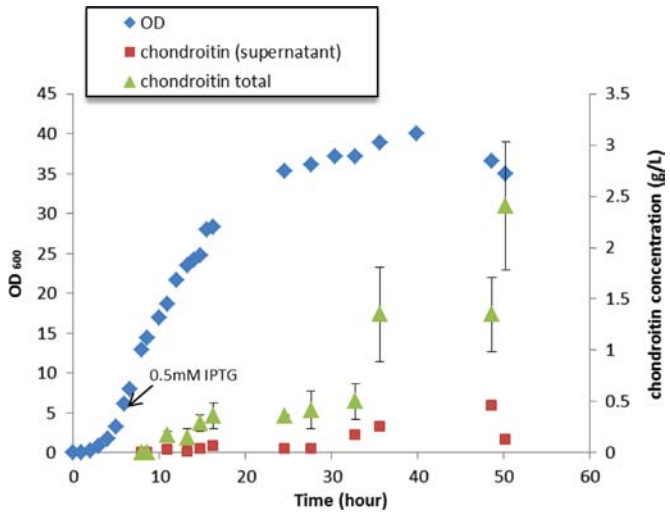


Fig. 8. Fermentation data for growth rate, production level of both intracellular and extracellular. The culture reached stationary phase after around 40 h while the maximum yield was observed at the maximum OD600 reading.

The other approach concerning the availability of UDP sugar may also affect the final yield of chondroitin. Theoretically, different gene orders in a pseudo-operon configuration can result in different expression levels of each gene. For example, for the pETM6_PACF construct, the RNA polymerase binds to the three T7 promoters driving the transcription of each of *kfoA*, *kfoC* and *kfoF* genes. However, each transcript ends at the same terminator, located downstream of the final gene. Therefore, it is expected that *kfoF* is most transcribed; *kfoC* is transcribed at intermediate level and *kfoA* is the least transcribed gene (Fig. 7). Since the chondroitin polymerase encoded by *kfoC* alternatively transfers UDP-GlcA and UDP-GalNAc to the non-reducing end of oligosaccharide/polysaccharide acceptor, it closely related to both pathways shown in Fig. 2. When the expression level of *kfoC* was high (red shaded in Table 2), the higher expression of *kfoA* compared to *kfoF* enhanced the overall production. The same phenomenon was also observed when *kfoC* had a medium level of expression. However, this trend was completely flipped when *kfoC* had the least expression level (blue shaded in Table 2). The first two observations are consistent with our hypothesis that overexpression of *kfoA* increases the availability of UDP-GalNAc and increases the overall chondroitin production. However, when expression of *kfoC* was limited, enzyme KfoF draws more carbon flux towards the UDP-GlcA synthesis pathway. Therefore, even with *kfoA* at a high level of expression, the overall availability of UDP-GalNAc may still be limited, thus resulting in lower amount of chondroitin production. Interestingly, this strategy yielded the highest chondroitin production level in a shake flask. One possible explanation is that increased carbon flux in the UDP-GlcA pathway hinders the UDP-GalNAc pathway. Therefore, it may further down-regulate the peptidoglycan synthesis pathway, which is the major competing pathway for UDP-GalNAc. At the same time, the expression of *kfoA* may allow more effective utilization of UDP-GalNAc to the chondroitin synthesis.

Furthermore, the intracellular and extracellular analysis of chondroitin in Fig. 7 may also shed some light on these results. Although most literature reports that *E. coli* BL21 Star™ (DE3) is capable of transporting all polysaccharide outside the cell (Andreishcheva and Vann, 2006), we found that a significant amount of chondroitin also accumulates inside the cell. The relative distribution of intracellular and extracellular chondroitin in all of the constructs is shown in Fig. 7. The pETM6-PFAC, pETM6-PACF, pETM6-PAFC and pETM6-PFCA constructs show approximately equal distribution of intracellular and extracellular chondroitin. One possible explanation is that the polysaccharide relocation mechanism is efficient. However, most of the transported polysaccharide was retained on the bacteria

Table 2
The impact of gene order of pseudo-operon structure on overall chondroitin production. The construct with pETM6_pCAF and pETM6_pCFA has the relative optimal yield compared to other order of genes. The production of chondroitin is detected from both inside the cell and the supernatant suggesting an insufficient transporting mechanism of polysaccharide in *E. coli* BL21 Star™ (DE3). The proposed expression intensity level is listed below. The blue square corresponds to the lowest expression level, the yellow square is the medium expression level and red square is the highest expression level. The similarity between the highest production construct is that they all have the least *kfoC* expression levels.

Chondroitin (mg/L)	***kfoF* low**	***kfoF* med**	***kfoF* high**
kfoA low	N/A	85.74	110.8
kfoA med	108.66	N/A	213.62
kfoA high	124.69	166.34	N/A

membrane surface, linked to poly-KDO linker (Cress et al., 2014). No gene was found in the genome of *E. coli* BL21 Star™ (DE3) that encodes a chondroitin lyase, responsible for polysaccharide shedding. Thus, the release of polysaccharide into the supernatant may be severely restricted. Another possible explanation may be that the transport mechanism is completely shut down in *E. coli* BL21 Star™ (DE3) and that the only chondroitin found in the supernatant comes from release of intracellular chondroitin through cell death.

Another interesting phenomenon is that a higher amount of chondroitin is formed during stationary phase, particularly close to the cell death phase, as the OD starts to drop. The synthesis of recombinant proteins requires both energy and a nitrogen source. When the metabolism of the cell adjusts to adopt the current environment and starts producing product, a lack of sufficient nitrogen may result in cell death. A complex nitrogen source was avoided in this study since these often can contain GAG. It will be essential to develop and optimize media composition for further improving the level of chondroitin production in future studies.

5. Conclusion

This study provides an alternative approach to produce chondroitin from non-pathogenic recombinant strain *E. coli* BL21 Star™ (DE3) by utilizing metabolic engineering. The maximum volumetric production reached 213 mg/l in shake flasks and 1.9 g/l in a 2-l fed batch fermenter. Both HPLC–MS and NMR studies confirmed the correct chemical structure of chondroitin, representing a clear improvement over the production of fructosylated chondroitin CPS by *E. coli* K4. Further studies on the transport mechanism of the polysaccharide, balancing the carbon flux of biosynthesis pathway, and optimizing the fermentation strategies should result in higher production levels. In addition to the potential industrial value, *E. coli* BL21 Star™ (DE3) also provides a more suitable platform for sulfation *in vivo*. This opens more opportunities for the metabolic engineering of sulfated chondroitins in recombinant *E. coli* BL21 Star™ (DE3) strain.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.11.003.

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