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Biosynthesis of Chondroitin in Engineered *Corynebacterium glutamicum* �

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Chondroitin, the precursor of chondroitin sulfate, which is an important polysaccharide, has drawn significant attention due to its applications in many fields. In the present study, a heterologous biosynthesis pathway of chondroitin was designed in a GRAS (generally recognized as safe) strain *C. glutamicum*. *CgkfoC* and *CgkfoA* genes with host codon preference were synthesized and driven by promoter P*tac*, which was confirmed as a strong promoter via GFPuv reporter assessment. In a lactate dehydrogenase (*ldh*) deficient host, intracellular chondroitin titer increased from 0.25 to 0.88 g/l compared with that in a wild-type host. Moreover, precursor enhancement via overexpressing precursor synthesizing gene *ugdA* further improved chondroitin titers to 1.09 g/l. Chondroitin production reached 1.91 g/l with the engineered strain *C. glutamicum* ΔL-*Cg*CAU in a 5-L fed-batch fermentation with a single distribution M_w of 186 kDa. This work provides an alternative, safe and novel means of producing chondroitin for industrial applications.

Keywords: Chondroitin biosynthesis, engineered *Corynebacterium glutamicum*, lactate dehydrogenase deficient, precursor enhancement, fed-batch fermentation

Introduction

Chondroitin sulfate (CS) is an essential glycosaminoglycan (GAG) located in mammalian extracellular matrices [1]. Due to its diverse biological and physiological functions, CS has been widely investigated and applied in clinical fields, such as an anti-inflammatory drug for treating osteoarthritis and rheumatism, in cartilage disease treatment, in cancer diagnoses and treatment, and tissue scaffold building with other biomacromolecules (*e.g.*, proteoglycan and hyaluronic acid) [2–4]. As the world population increasingly ages, the market demand for CS has correspondingly increased.

CS is conventionally extracted from animal tissues, such as shark fins and bovine trachea. While seemingly industrially feasible, the reliance on an animal-derived production process has a number of drawbacks, including low productivity, costly downstream processing and harmful environmental impacts [5]. Additionally, there are growing concerns for potential risks of interspecies viral transmission. In response, researchers have turned to microbial production as a safer and more reliable alternative. Chondroitin, containing β-D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc) as a disaccharide unit, is the backbone of CS and shares structural similarity with other GAGs (Fig. S1). Chemo-enzymatic sulfation of microbially produced chondroitin has become an attractive approach to producing CS [6–9].

A capsular polysaccharide (CPS) of *Escherichia coli* K4 has been discovered as an unsulfated chondroitin with a fructose residue at the 3-position of GlcUA [10]. The similarity of K4CPS (fructose chondroitin) to chondroitin allows for potential CS production by microbial fermentation. Through defructosylation and sulfation, K4CPS could be further converted into CS [11]. Researchers have explored the biochemical basis of fructose chondroitin synthesis in bacteria and have identified enzymes involved in this process [6]. Cimini *et al.* enhanced K4CPS production

by genetic engineering and bioprocess optimization. They achieved K4CPS titers of 3.5 g/l by replacing transposase with chondroitin polymerase [12]. Recently, Wu *et al*. attempted to balance the metabolic flux of intracellular K4CPS precursors UDP-GalNAc and UDP-GlcUA and obtained an 8.4 g/l titer (the highest level achieved to date) in a 30-L fermentor by glycerol and dissolved oxygen-stat feeding [13]. Despite these promising results, *E. coli* K4 is a pathogenic bacteria and may cause urinary tract infections [14]. Thus, an alternative chondroitin production pathway in a safer host was considered.

He *et al*. constructed a plasmid system with a pseudo-operon containing gene *kfoC-kfoA-kfoF* in *E. coli* BL21, and achieved a 2.4 g/l intracellular chondroitin titer in a dissolved oxygen-stat fed-batch bioreactor [15]. *Bacillus subtilis*, regarded as a GRAS (generally recognized as safe) strain, is also an alternative in the heterogeneous production of chondroitin. Jin *et al*. utilized *B. subtilis* 168 as a host and successfully produced extracellular chondroitin by integrating expression cassette P*xylA-kfoC-kfoA* into the genome and enhanced the biosynthesis of chondroitin by *tuaD* upregulation. Titers of 2.54 g/l and 5.22 g/l extracellular chondroitin were accumulated in shaking flask culture and 3-L fermentor, respectively. The weight-average molecular weights (M_w) of these products were 114.07 kDa in the shaking flask and 65.93 kDa in the 3-L fermentor. Although the results showed that *B. subtilis* 168 was able to transport chondroitin to the extracellular matrix, the mechanism by which this occurred remained unclear [16]. Zhou *et al*. further compared operon structures among different enzymes responsible for the biosynthesis of chondroitin precursors and obtained 7.15 g/l chondroitin in engineered *B. subtilis* 168 after 70 h cultivation in a 3-L fermentor [17].

Corynebacterium glutamicum is a Gram-positive strain free of exotoxins and endotoxins. As a GRAS strain, it is an excellent host to produce food and drug related products, such as organic acids and amino acids [18–21]. In our previous study, *C. glutamicum* was engineered to efficiently biosynthesize hyaluronic acid (HA), a GAG similar to chondroitin [22, 23]. Moreover, *C. glutamicum* is a nonsporulating strain that will not suffer from spore formation at the late stage of fermentation as *B. subtilis*.

In the present research, we attempted to biosynthesize chondroitin in the novel host *C. glutamicum* via several strategies, *i.e.*, pathway engineering, promoter optimization, byproduct gene deletion and precursor upregulation. Finally, a fed-batch culture of recombinant *C. glutamicum* was conducted in a 5-L fermentor to produce chondroitin.

Material and Methods

DNA Manipulation

Plasmid DNA, agarose gel electrophoresis, restriction enzyme digestion, DNA ligation and DNA transformation were performed following standard protocols [24] or manufacturer instructions. Phanta DNA polymerase used in PCR was purchased from Vazyme Biotech Co., Ltd. (China). Gel extraction kit and plasmid miniprep kits were purchased from Omega. QuickCut restriction enzymes were purchased from Takara.

Gene, Plasmid Vector and Bacterial Strain

Gene cluster C*gkfoC-CgkfoA* containing RBS sequence was synthesized by Qinglan Biotech Co., Ltd. (Wuxi, China), with *XbaI/KpnI* restriction sites using primers C-F and A-R. Gene *ugdA* was cloned from the genome of *C. glutamicum* ATCC13032 using primers U-F and U-R with the restriction sites *KpnI/SacI*. Table S1 in Supporting Information lists all primers used in this study.

Plasmid pXMJ19 harboring inducible promoter P*tac* served as the backbone of the vector. All recombinant plasmids were constructed by inserting the target genes into the vector and are summarized in Supporting Information, Table S2.

C. glutamicum-Δldh was constructed via double crossover homologous recombination driven by negative selection marker gene *sacB* [25]. Both *C. glutamicum* ATCC13032 and *C. glutamicum-Δldh* were used for expressing the chondroitin biosynthesis operon. *E. coli* Top 10 (Solarbio) was used for cloning a shuttle vector and its driven plasmids. All wild-type and engineering strains are summarized in Supporting Information, Table S2.

Codon Usage Preference Analysis

The codon usage preference of *C. glutamicum* was based on the Kazusa online database (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=196627&aa=1&style=N). Statistical analyses of the rare codons were conducted via a graphical codon usage analyzer (http://gcua.schoedl.de/sequential_v2.html).

Chondroitin Titer and Weight-Average Molecular Weight Measurement

Fermentation broth was centrifuged at 4°C and 10,000 ×*g* to separate cell pellets and supernatant. To determine intracellular chondroitin concentration, cell pellets were collected and redissolved in deionized water. Then, the intracellular contents were released via an Ultra-High Pressure Continuous Flow Cell Disrupter JN-02C (JNBIO, Guangzhou, China). The supernatant was collected and centrifuged at 4°C, 10,000 ×*g* to remove cell fragments. To purify the chondroitin, intracellular supernatant was precipitated using 3 volumes of ethanol and incubated at 4°C for 3 h. The recovered sediments were redissolved in deionized water for further chondroitin titer measurement. To determine extracellular chondroitin concentrations, supernatants from the fermentation broth were mixed with 3 volumes of ethanol to precipitate chondroitin at 4°C for 3 h. Precipitated chondroitin

was redissolved in deionized water for further chondroitin titer measurement.

A modified uronic acid carbazole assay [26] was applied to determine intracellular and extracellular chondroitin titers. Briefly, a 0.5 ml sample was added to 3 ml sulfuric acid reagent (9.5 g/l sodium tetraborate dissolved in sulfuric acid) and heated in boiling water for 20 min. Then, 0.1 ml carbazole reagent (1.25 g/l carbazole dissolved in ethanol) was added and heated for another 15 min. Finally, OD_{530} was measured to determine the chondroitin titer according to the standard curve. *C. glutamicum* with empty plasmid was used as a blank control.

Gel permeation chromatography (GPC) combined with a differential refraction detector was applied to measure chondroitin M_w. Monodisperse HA was used as a standard; these details have been reported in our previous study [22].

Chondroitin Digestion by Chondroitinase ABC and Mass Spectra Analysis

To digest chondroitin, 1 ml product solution (1 mg/ml) was treated with 1 IU of chondroitinase ABC (Bicheng Biotech Co., Ltd., China) at 25 °C. Triple quadrupole liquid chromatography-mass spectrometry (Shimadzu, Japan) was applied to analyze the disaccharide unit with electrospray ionization mass spectrometry (ESI-MS) in negative scan mode. MS/MS negative scan mode was further used to analyze monosaccharide fragments from peak m/z = 378.

Shaking Flask Culture of Recombinant *C. glutamicum*

Seed culture was conducted in LBG20 (yeast extract 5 g/l, peptone 10 g/l, NaCl 10 g/l, and glucose 20 g/l) liquid medium with 5 μg/ml chloramphenicol at 30°C, 200 rpm overnight. When the OD_{600} reached 2.5, 5% v/v inoculums were added to a 300-ml flask containing 50 ml modified culture medium [22] (glucose 40 g/l, corn syrup powder 20 g/l, $(NH_4)_2SO_4$ 30 g/l, KH_2PO_4 1 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 5 g/l, $FeSO_4•7H_2O$ 10 mg/l, and $MnSO_4•7H_2O$ 10 mg/l) with 5 μg/ ml chloramphenicol. Shaking flask culture was performed at 28°C, 200 rpm, and 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added at 3 h to induce gene expression. *C. glutamicum* containing empty plasmid pXMJ19 was used as a control.

GFPuv Reporter Assessment

Six engineered strains containing different promoters (*i.e.*, P*ddh*, P*dapA*, P*sod*, P*fba*, P*tuf*, and P*tac*) were constructed with pEC-XK99E plasmid for promoter strength analysis. UV light-excited green fluorescent protein (GFPuv) was used as a reporter gene [27]. Briefly, seed cultures of six recombinant strains were conducted in LBG20 liquid medium with 50 μg/ml kanamycin at 30°C, 200 rpm overnight. Then, 5% v/v inoculums were added to the 300-ml flask containing 50 ml modified culture medium [19] (for P*tac*-GFPuv, 1 mM IPTG was added at 3 h). After 24 h, cells were harvested at 4°C, 10,000 ×*g*. The pellets were resuspended in deionized water to OD_{600} = 1.0 and GFPuv expression intensities were monitored on a TECAN Infinite M200 PRO microplate reader (Männedorf, Switzerland). Wild-type *C. glutamicum* ATCC13032 was used as a control. Absorbance was measured at 385 nm and 509 nm extinction and emission wavelengths, respectively. The native promoter sequences were referenced from a previous promoter study [28].

SDS-PAGE of Target Protein

The 24 h fermentation sample taken from the shaking flask culture was centrifuged at 4°C, 10,000 ×*g* to collect cell pellets. The cell disrupter described earlier was used to release the whole cell proteins of recombinant *C. glutamicum*. Then, the sample was loaded onto Coomassie-stained SDS-PAGE to verify target protein expression.

RNA Isolation, Reverse Transcriptase PCR and Real-Time Quantitative PCR (qRT-PCR)

Cell pellets were collected from shaking flask culture at 24 h when the recombinant strains were in mid-exponential phase. An E.Z.N.A. Bacterial RNA Kit (Omega, USA) was applied to extract total RNA and reverse transcription using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, China) to generate a cDNA library. Target gene transcript levels were quantified by qRT-PCR using the ABI 7300 real-time PCR system (Applied Biosystems, USA) and AceQ qPCR SYBR Green Master Mix (Vazyme, China) using primers qU-F and qU-R. Gene dnaE was used to normalize the target gene transcription level using qE-F and qE-R. Each experiment was performed in triplicate. Samples without reverse transcriptase treatment were used as negative controls to eliminate interference caused by DNA contamination.

Fed-Batch Culture in a 5-L Fermentor

A fed-batch culture of engineered *C. glutamicum* was performed in a 5-L fermentor (Sartorius stedim, BIOSTAT B plus) with a 2-L working volume. Aeration and agitation were set at 1 vvm and 600 rpm, respectively. Briefly, 6 M NaOH and 6 HCl were used to maintain fermentation pH at 7.2. A 5% v/v inoculum was prepared, and the fermentation temperature was controlled at 28°C. At 3 h, 1 mM IPTG was added to induce gene expression.

Results

Construction of Chondroitin Biosynthesis Pathway in *C. glutamicum*

Based on the biosynthesis mechanism of chondroitin in *E. coli* K4, we designed a biosynthesis pathway in *C. glutamicum* as shown in Fig. 1. Heterologous UDP-N-acetylglucosamine-4-epimerase (KfoA) and chondroitin polymerase (KfoC) from *E. coli* K4 were introduced into the engineered *C. glutamicum* to generate the extra precursor UDP-GalNAc. Then, the chondroitin polymer was fabricated with another native precursor UDP-GlcUA.
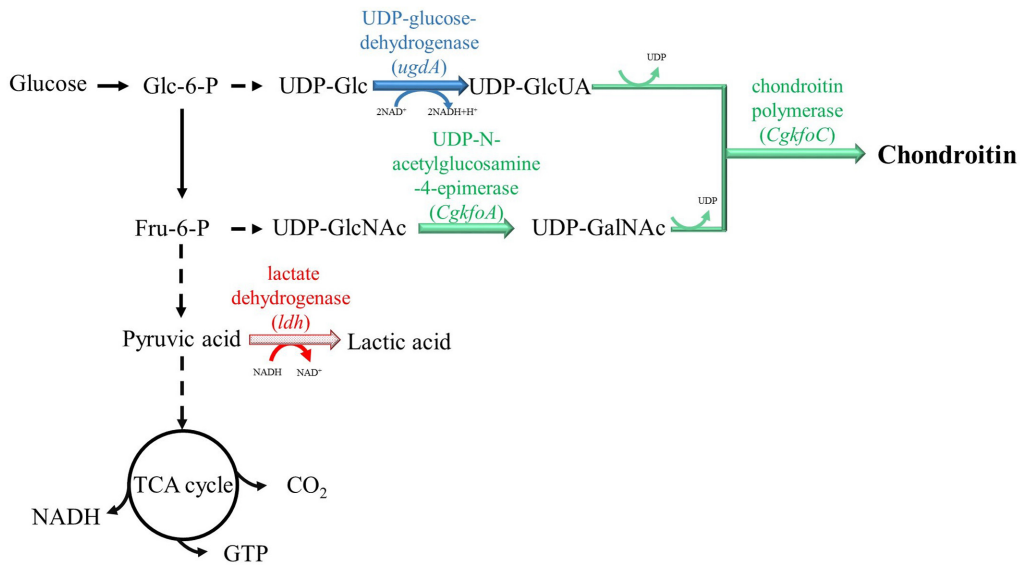
Fig. 1. Chondroitin biosynthesis pathway starting from glucose in recombinant *C. glutamicum*.
Heterologous genes (*CgkfoC* and *CgkfoA*) are marked in green bold arrow. Native gene (*ugdA*) is marked in blue bold arrow. Byproduct synthesis gene (*ldh*) is marked in red dot arrow. Dashed line represents multiple enzyme reactions.

Codon usage preference analyses showed that the original *E. coli* K4 *kfoC* gene (687 codons) contained 37 rare codons (usage frequency, 10–20%) and 38 highly rare codons (usage frequency < 10%) of *C. glutamicum*. The *E. coli* K4 *kfoA* (340 codons) contained 21 rare codons and 17 highly rare codons of *C. glutamicum* (Fig. S2). To eliminate any possible negative effects from these rare codons, two new genes, *CgkfoC* and *CgkfoA,* were designed and synthesized

with a codon preference for *C. glutamicum* (Fig. S3).

The promoter is another key factor determining the overexpression efficiency of heterologous genes. Using the same plasmid vector, five native promoters (P*ddh*, P*dapA*, P*fba*, P*sod*, and P*tuf*) and one inducible strong promoter P*tac* were compared with green fluorescent protein as a reporter. An optimal *C. glutamicum* RBS, AAAGGAGGA [29], was also applied for GFPuv overexpression. As shown
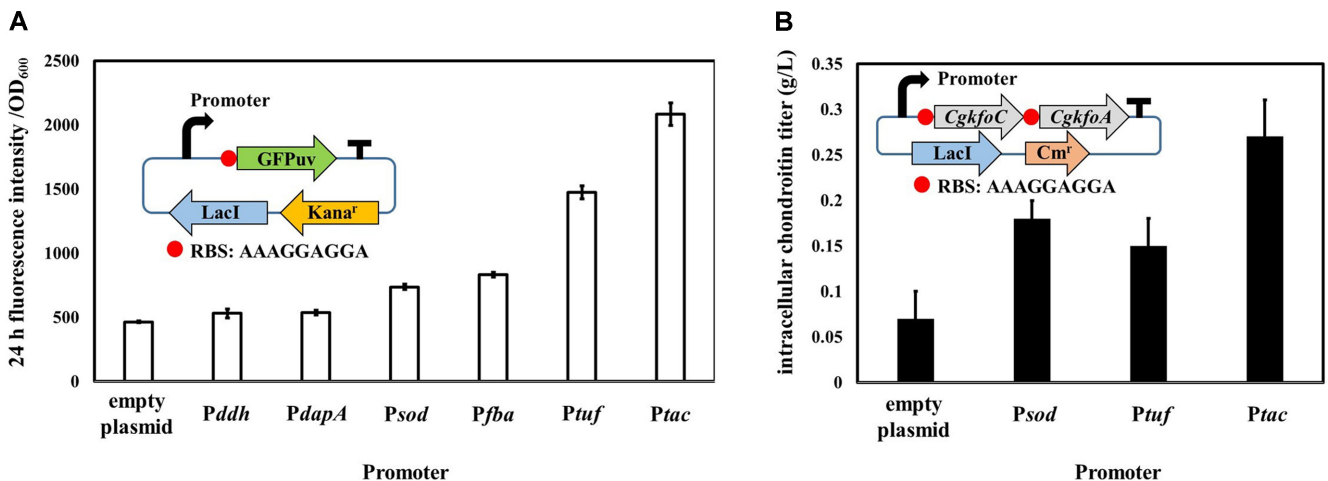

Fig. 2. (**A**) GFPuv reporter analysis of 5 native promoters and one inducible promoter P*tac* in *C. glutamicum*. P*ddh*, P*dapA*, P*sod*, P*fba,* and P*tuf*, promoters of diaminopimelate dehydrogenase, 4-hydroxy-tetrahydrodipicolinate synthase, superoxide dismutase, fructose 1,6 bisphosphate aldolase and translational elongation factor EF-Tu. P*tac*, chimeric promoter of *E. coli*. (**B**) Chondroitin titers of recombinant *C. glutamicum* harboring *CgkfoC* and *CgkfoA* driven by P*tac*, P*tuf,* and P*sod*.

in Fig. 2A, P*tac* and P*tuf* were strong promoters, and P*sod* and P*fba* were moderately strong promoters in *C. glutamicum*. Furthermore, plasmid pXMJ19 harboring P*tac*-*Cg*KfoCA, P*tuf*-*Cg*KfoCA and P*sod*-*Cg*KfoCA were transformed into *C. glutamicum*. Their chondroitin titers were measured with empty plasmid strain (Fig. 2B). Thus, inducible promoter P*tac* was an ideal promoter for expressing the *kfo* genes in recombinant *C. glutamicum.*

Lactic acid synthesis, catalyzed by lactate dehydrogenase (LDH), dissipates the energy produced from GAG production. In our previous study, deletion of gene *ldh* effectively increased ATP and carbon flux redistribution to GAG production [23]. Herein, gene *ldh* knockout was applied (yielding *ldh*-knockout strain *C. glutamicum-Δldh*) to evaluate and compare its impact on chondroitin synthesis with wild-type host *C. glutamicum* ATCC 13032. The recombinant plasmid pXMJ19-P*tac*-*Cg*KfoCA was transformed into both *C. glutamicum* ATCC 13032 and *C. glutamicum-Δldh*, yielding the engineered strains *C. glutamicum-Cg*KfoCA (*Cg*CA) and *C. glutamicum-Δldh-Cg*KfoCA (ΔL-
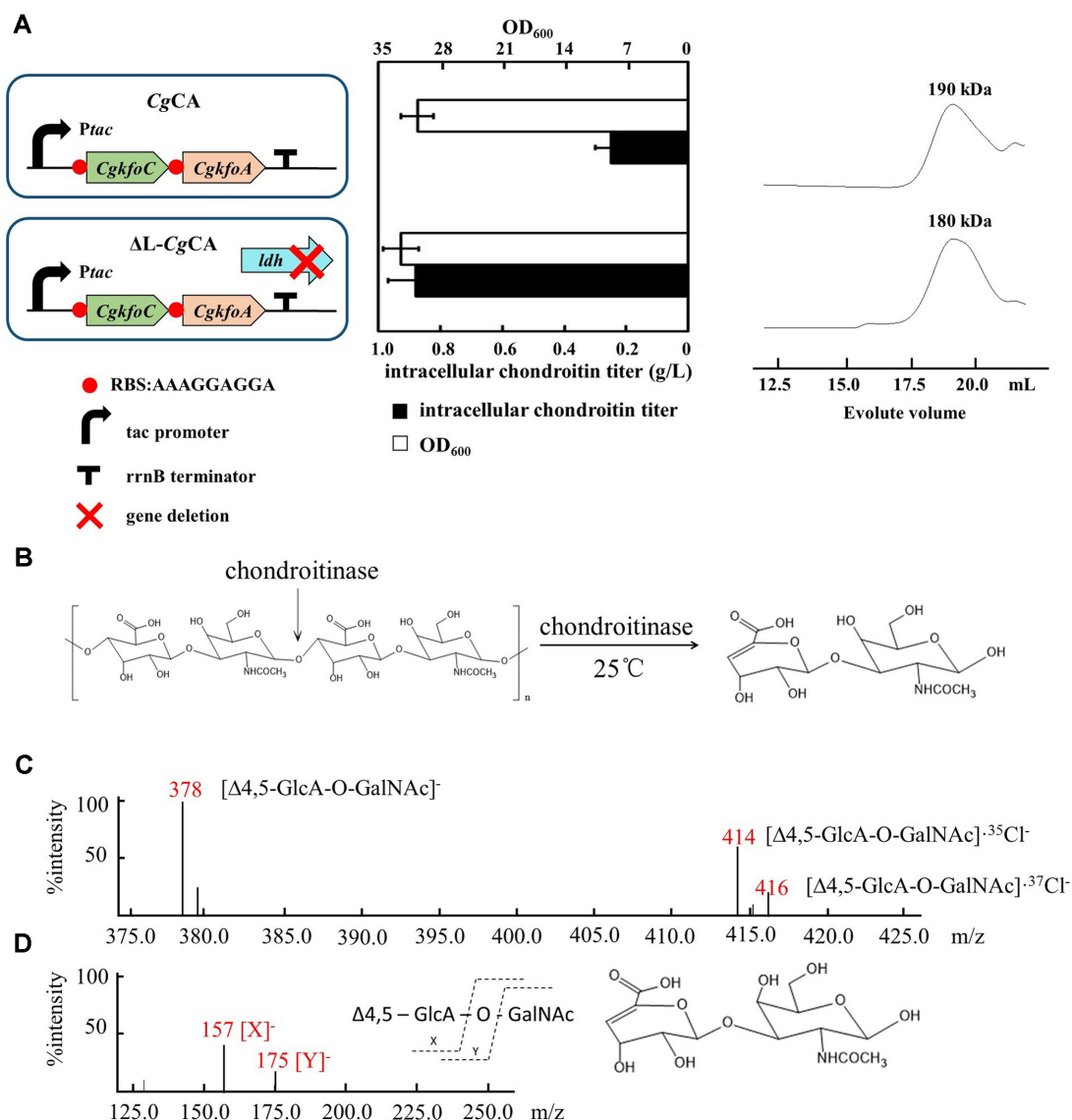

Fig. 3. Chondroitin biosynthesis in recombinant *C. glutamicum Cg*CA and ΔL-*Cg*CA. (**A**) Intracellular chondroitin titer, OD_{600} and M_w of control strain (with empty plasmid), *Cg*CA and ΔL-*Cg*CA in shaking flask culture. (**B**) Chondroitinase ABC hydrolysis reaction. (**C**) ESI-MS result of the digested chondroitin product in negative scan mode. (**D**) MS/MS result of peak m/z = 378 in negative scan mode. The target peaks were labeled with its m/z and molecular formula. Each experiment was performed in triplicate.

*Cg*CA). Successful expressions of KfoC and KfoA in *C. glutamicum* were verified by SDS-PAGE (Fig. S4).

Shaking flask culture was performed in modified medium to investigate the chondroitin production capability of *Cg*CA and ΔL-*Cg*CA. As shown in Fig. 3A, the engineered strain *Cg*CA accumulated 0.25 g/l intracellular chondroitin, whereas the *ldh*-knockout significantly increased the chondroitin titer to 0.88 g/l with nearly the same M_w (180-190 kDa). The molecular structure of chondroitin was verified by chondroitinase ABC digestion (Fig. 3B) and subsequent ESI-MS (Fig. 3C) and MS/MS (Fig. 3D) analyses of the digested products. As shown in Fig. 3C, disaccharide units [Δ4,5-GlcA-O-GalNAc]⁻, [Δ4,5-GlcA-O-GalNAc]$^{.35}$Cl⁻ and [Δ4,5-GlcA-O-GalNAc]$^{.37}$Cl⁻ were identified. As shown in Fig. 3D, two monosaccharide fragments were observed by MS/MS. No extracellular chondroitin was obtained, however, indicating that the extracellular transport of chondroitin was restricted in *C. glutamicum*.

Enhanced Biosynthesis of Chondroitin via *ugdA* Overexpression

According to studies on the microbial production of GAG (*e.g.*, hyaluronic acid and chondroitin), UDP-glucose dehydrogenase was identified as a rate-limiting factor in the synthesis pathway [12, 16, 23, 30]. Gene *ugdA* expression was up-regulated to enhance the biosynthesis of UDP-GlcUA, another chondroitin precursor (Fig. 1). The native *ugdA* of *C. glutamicum* was cloned and integrated into pXMJ19-P*tac*-*Cg*KfoCA, yielding pXMJ19-P*tac*-*Cg*KfoCAU. Recombinant plasmid pXMJ19-P*tac*-*Cg*KfoCAU was transformed into wild-type and *ldh*-knockout strains to obtain two new strains: *C. glutamicum*-*Cg*KfoCAF (*Cg*CAU) and *C. glutamicum*-Δ*ldh*-*Cg*KfoCAU (ΔL-*Cg*CAU) (Fig. 4A). The qRT-PCR results confirmed that the transcript levels of *ugdA* in *Cg*CAU and ΔL-*Cg*CAU were significantly enhanced (over 20-fold after accounting for DNA interference) after *ugdA* overexpression with P*tac* promoter (Fig. 4B). Chondroitin
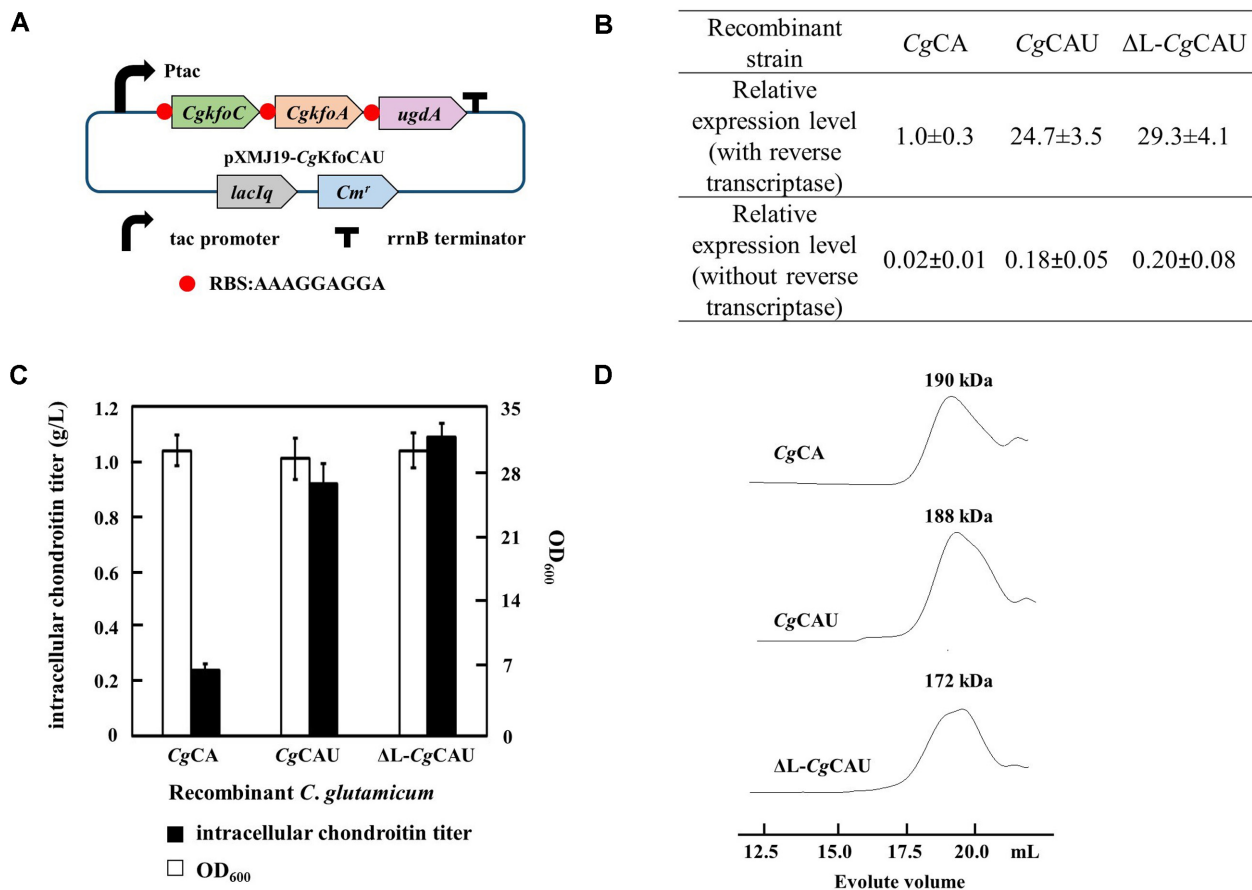

Recombinant strain	*Cg*CA	*Cg*CAU	ΔL-*Cg*CAU
Relative expression level (with reverse transcriptase)	1.0±0.3	24.7±3.5	29.3±4.1
Relative expression level (without reverse transcriptase)	0.02±0.01	0.18±0.05	0.20±0.08

Fig. 4. Chondroitin biosynthesis in *ugdA* overexpressed recombinant *C. glutamicum Cg*CAU and ΔL-*Cg*CAU.
(**A**) Plasmid structure of pXMJ19-Cg*Kfo*CAU (**B**) qRT-PCR results of gene *ugdA* transcription in different engineered strains. (**C**) Intercellular chondroitin titer and OD$_{600}$ of *Cg*CA, *Cg*CAU and ΔL-*Cg*CAU. (**D**) Chondroitin's M_w. Experiments were performed in triplicate.

titers from these two strains were compared in shaking flask culture using *CgCA* as a control. As illustrated in Fig. 4C, the overexpression of *ugdA* in a wild-type host increased the chondroitin titer from 0.24 g/l to 0.92 g/l, whereas in the *ldh* deletion strain ΔL-*CgCAU*, the chondroitin titer was 1.09 g/l (Fig. 4C). Similar M_w distributions (172, 188, and 190 kDa, respectively) were also observed for the engineered strains (Fig. 4D). Collectively, strain ΔL-*CgCAU* showed the highest chondroitin titer and thus, was chosen for further investigation in the fed-batch culture.

Fed-Batch Culture of Recombinant ΔL-*CgCAU* in a 5-L Fermentor

Because chondroitin consists of multiple disaccharide units, glucose was fed to ensure a sufficient carbon source for chondroitin production. As shown in Fig. 5A, we continuously pumped glucose into the fermentor to maintain glucose concentration at 8–15 g/l from 17 h until the end of the cultivation (Fig. 5A).

During the fed-batch culture, a maximum OD_{600} of recombinant strain ΔL-*CgCAU* reached 101, indicating the start of the stationary phase at 20 h (Fig. 5B). The maximum chondroitin titer reached 1.91 g/l at 28 h. Productivity was calculated as 0.068 g/l/h at this time. GPC analysis showed that the M_w of the chondroitin product remained stable at approximately 186 kDa throughout the fermentation (Fig. 5C).

Discussion

With the development of biotechnology and increasing health concerns regarding animal-derived products, the microbial production of chondroitin shows great potential for producing CS. A safer but efficient host is an important property for the microbial production of chondroitin. As reported in the literature, engineered *B. subtilis* 168 has been the most successful host in producing chondroitin [16, 17]. In this work, however, *C. glutamicum* was utilized for the first time, and its capability to produce chondroitin was explored via several engineered strategies.

The chondroitin synthesis pathway of *E. coli* K4 was mimicked. Two key enzymes, KfoC and KfoA for synthesizing chondroitin, were overexpressed in *C. glutamicum*. Two novel genes, *CgkfoC* and *CgkfoA*, were synthesized after codon preference optimization. The optimal strong promoter, P*tac*, was selected for the overexpression of *CgkfoC* and *CgkfoA* and compared with 5 other widely used promoters, such as P*tuf* and P*sod* of *C. glutamicum*; P*tuf* is generally
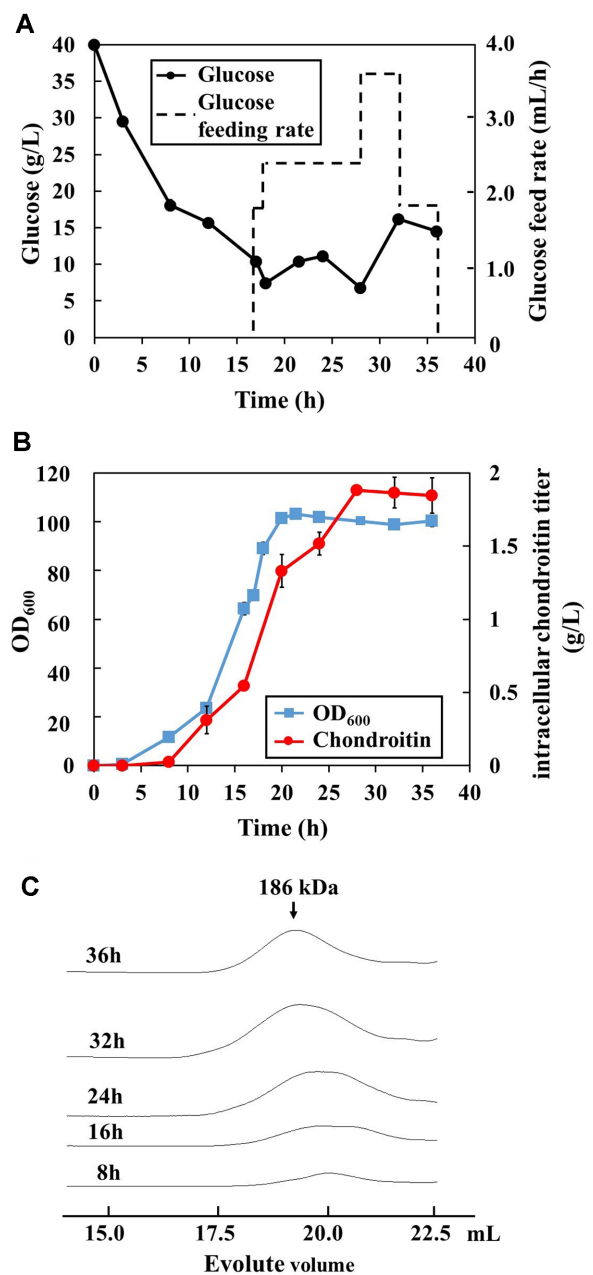
Fig. 5. Fed-batch culture of ΔL-*CgCAU* in a 5-L fermentor.
(**A**) Glucose concentration (solid line) and feeding rate (dashed line). (**B**) OD_{600} and chondroitin titer. (**C**) M_w of chondroitin. Experiments were performed in duplicate.

acknowledged as a strong promoter [28, 31]. The GFPuv reporter assessment results showed that P*tac* was more efficient than the native strong promoter P*tuf* [28]. Furthermore, *CgkfoC* and *CgkfoA* co-expression driven by P*tac* generated 0.25 g/l chondroitin in the engineered *C. glutamicum*.

Carbon flux redirection is an effective method for intensifying GAG production. For instance, HA titer was promoted via *ldh* deletion in *L. lactis* and *C. glutamicum* [23, 32]. Its effect also embodied in redox balance [32] and enhancing ATP supplementation [23]. Herein, an LDH knockout *C. glutamicum* host was applied to enhance chondroitin production. The chondroitin titer increased to 0.88 g/l, illustrating that deleting *ldh* was effective to enhance chondroitin biosynthesis.

An over-expressing of the enzymes responsible for the biosynthesis of UDP-sugar precursors from carbon source is another major approach for enhancing GAG production. Previous studies have demonstrated that UDP-glucose dehydrogenase (KfoF in *E.coli* K4, KfiD in *E.coli* K5, HasB in *S. equi*, TuaD in *B. subtilis* and UgdA in *C. glutamicum*) in the GAG biosynthesis pathway is a rate-limiting enzyme and is strictly regulated in UDP-GlcUA biosynthesis [22, 33]. For example, in a study of hyaluronic acid production by recombinant *C. glutamicum*, the optimal operon structure was *hasA-hasB* [22, 23]. Similar strategies have also been applied in the heterogeneous production of heparson, another GAG with a similar structure as HA and chondroitin [16]. The overexpression of *tuaD* (an encoding isozyme of KfoF) in recombinant *B. subtilis* also resulted in a promotion of chondroitin titer from 1.63 g/l to 2.36 g/l [16]. In this study, precursor UDP-GlcUA was enhanced via the overexpression of *ugdA* with strong promoter P*tac*. The chondroitin titer was enhanced more than 3-fold, indicating that UDP-glucose dehydrogenase is a rate-limit enzyme as well in chondroitin biosynthesis of engineered *C. glutamicum*.

Molecular weight is another important property of microbial-produced chondroitin. In the study of recombinant *B. subtilis*, the M_w of chondroitin increased from 84 kDa to 114 kDa after UDP-glucose dehydrogenase upregulation, but decreased to 66 kDa after the culture mode changed from shaking flask to 3-L fermentor. Jin et al. attributed the decrease to shearing forces caused by mechanical agitation [16]. In this work, however, the M_w from different strains or different culture modes were all approximately 180 kDa. Because nearly all chondroitin products were retained in the cell pellet, the cell membrane and walls may shield chondroitin from external factors. Due to the negative relation between GAG titer and its M_w in HA production [34, 35], this high M_w may contribute to the relatively low chondroitin titer in the engineered *C. glutamicum*.

Further research on chondroitin titer enhancement is still required. A more comprehensive operon structure that takes both UDP-GalNAC and UDP-GlcUA into consideration and an efficient transport system that can obtain extracellular chondroitin or other metabolic or transcriptional regulations are feasible strategies for engineered *C. glutamicum*.

Acknowledgment

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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