Synthetic and Systems Biotechnology 9 (2024) 713–722

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Combinatorial metabolic engineering of *Escherichia coli* for *de novo* production of structurally defined and homogeneous Amino oligosaccharides

Jinqi Shi [a], Chen Deng [a,c], Chunyue Zhang [a], Shu Quan [a], Liqiang Fan [a,c,**], Liming Zhao [a,b,c,*]

[a] State Key Laboratory of Bioreactor Engineering, School of Biotechnology, East China University of Science and Technology, Shanghai, 200237, China
[b] Organ Transplant Center, Shanghai Changzheng Hospital, Shanghai, 200003, China
[c] Shanghai Collaborative Innovation Center for Biomanufacturing Technology (SCICBT), Shanghai, 200237, China

ARTICLE INFO

Keywords:
Amino oligosaccharides
Chitin oligosaccharides
Metabolic engineering
Fed-batch cultivation bioproduction

ABSTRACT

Amino oligosaccharides (AOs) possess various biological activities and are valuable in the pharmaceutical, food industries, and agriculture. However, the industrial manufacturing of AOs has not been realized yet, despite reports on physical, chemical, and biological approaches. In this study, the *de novo* production of chitin oligosaccharides (CHOS), a type of structurally defined AOs, was achieved in *Escherichia coli* through combinatorial pathway engineering. The most suitable glycosyltransferase for CHOS production was found to be NodCL from *Mesorhizobium Loti*. Then, by knocking out the *nagB* gene to block the flow of N-acetyl-D-glucosamine (NAG) to the glycolytic pathway in *E. coli* and adjusting the copy number of NodCL-coding gene, the CHOS yield was increased by 6.56 times. Subsequently, by introducing of UDP-N-acetylglucosamine (UDP-GlcNAc) *salvage* pathway for and optimizing fermentation conditions, the yield of CHOS reached 207.1 and 468.6 mg/L in shake-flask cultivation and a 5-L fed-batch bioreactor, respectively. Meanwhile, the concentration of UDP-GlcNAc was 91.0 mg/L, the highest level reported in *E. coli* so far. This study demonstrated, for the first time, the production of CHOS with distinct structures in plasmid-free *E. coli*, laying the groundwork for the biosynthesis of CHOS and providing a starting point for further engineering and commercial production.

1. Introduction

Amino oligosaccharides (AOs) are a specific type of oligosaccharides characterized by their degree of polymerization (DP), which ranges from 2 to 10. These AOs are formed by linking N-acetyl-D-glucosamine (NAG) or D-glucosamine (GlcN) molecules together [1]. Based on their structural variations, AOs can be categorized into two groups: homogeneous AOs and heterogeneous AOs [2,3]. Homogeneous AOs consist of a single type of NAG or GlcN unit, while heterogeneous AOs are composed of a combination of NAG and GlcN units. The distinctive structures of AOs, including their DP, degree of deacetylation, and molecular mass, contribute to their unique biological activities and functional mechanisms. AOs with different structural characteristics exhibit specific biological properties and functional mechanisms [4].

Chitin oligosaccharides (CHOS) and chitooligosaccharides (COS) are among the most extensively utilized AOs. Their applications span diverse fields, including food, medicine, cosmetics, and agriculture. CHOS consist of β-1,4-linked NAG, while COS consist of β-1,4-linked GlcN, with a small proportion of NAG, and both have a DP below 10 (Fig. 1A). Comparatively, CHOS and COS exhibit several advantages over chitin and chitosan, including lower molecular mass and enhanced solubility, leading to more comprehensive and superior biological activities. These biological activities encompass a wide range of functions, such as plant growth regulation, antioxidant properties, anti-inflammatory effects, antibacterial capabilities, anti-tumor properties, blood pressure and blood sugar regulation, modulation of gut microbiota, and immune system enhancement [5–8].

The production of AOs commonly involves chemical, physical, and enzymatic degradation of chitin. However, each method presents certain drawbacks. Chitin, typically sourced from discarded marine organisms,

Peer review under responsibility of KeAi Communications Co., Ltd.
* Co-corresponding author. School of Biotechnology, East China University of Science and Technology, Meilong Road 130, Shanghai, 200237, China.
** Co-corresponding author. School of Biotechnology, East China University of Science and Technology, Meilong Road 130, Shanghai, 200237, China.
E-mail addresses: fanglq@ecust.edu.cn (L. Fan), zhaoliming@ecust.edu.cn (L. Zhao).

https://doi.org/10.1016/j.synbio.2024.05.011
Received 8 March 2024; Received in revised form 2 May 2024; Accepted 20 May 2024
Available online 21 May 2024

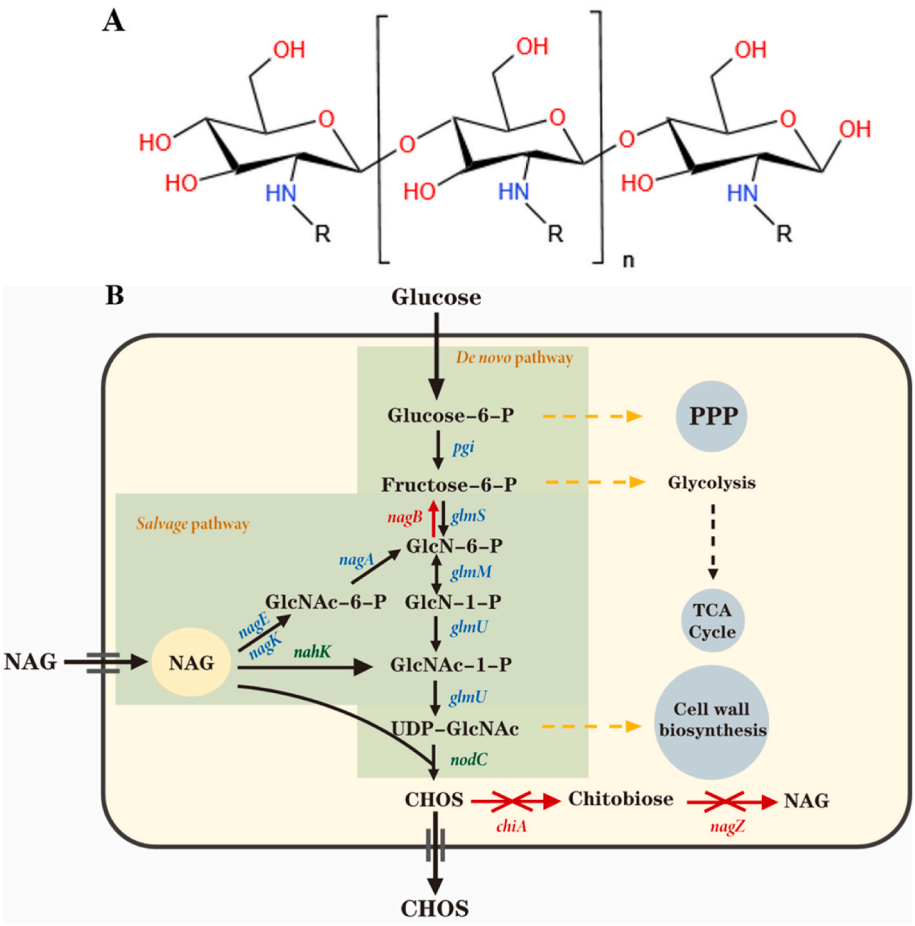

Fig. 1. Chemical structures of AOs and the metabolic pathway for CHOS biosynthesis in engineered *E. coli*. (A) Chemical structures of AOs. COS: R = H, n is the number between 2 and 8; CHOS: R = $-OCH_3$, n is the number between 0 and 8. (B) Metabolic pathway of CHOS biosynthesis in *E. coli*. The black arrows indicate: single reactions, and red arrows represent a blocking reaction. Metabolite abbreviations: Glucose-6-P, Glucose-6-phosphate; Fructose-6-P, Fructose-6-phosphate; GlcN-6-P, glucosamine-6-phosphate; GlcNAc-6-P, N-acetylglucosamine-6-phosphate; GlcN-1-P, glucosamine-1-phosphate; NAG, N-acetyl-D-glucosamine; GlcNAc-1-P, N-acetylglucosamine-1-phosphate. UDP-GlcNAc, uridine 5′-diphospho-N-acetylglucosamine; CHOS, chitin oligosaccharides. Genes abbreviations: *pgi*, encoding phosphoglucose isomerase; *glmS*, glucosamine-6-phosphate synthetase; *glmM*, encoding phosphoglucosamine mutase; *glmU*, encoding N-acetylglucosamine-1-phosphate uridyltransferase; *nagB*, encoding glucosamine-6-phosphate deaminase; *nagA*, encoding N-acetylglucosamine-6-phosphate deacetylase; *nagE*, encoding the GlcNAc-specific phosphotransferase system transporter; *nagK*, encoding N-acetyl-L-glutamate kinase; *nahK*, encoding N-acetylhexosamine 1-kinase; *nodC*, encoding N-acetylglucosaminyltransferase glycosyltransferase; *chiA*, encoding chitinase; *nagZ*, encoding β-N-acetylglucosaminidase. Other abbreviations: PPP, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle.

renders AOs obtained from chitin degradation unsuitable for individuals with seafood allergies. Furthermore, the random deacetylation of acetyl groups in NAG and irregular cleavage of glycosidic bonds in chitin during the degradation process result in AOs with varying structures. Additionally, the chemicals employed in the degradation process may contribute to environmental pollution and introduce impurities into the final product [9,10]. Physical degradation techniques can also cause alterations in the oligosaccharide structure [11,12]. Therefore, it is crucial to develop and implement novel technologies and methods that allow for the efficient and reliable production of structurally defined AOs.

Synthetic biology has emerged as a powerful approach to the synthesis of rare natural products and novel compounds. Among the chassis cells commonly employed in synthetic biotechnology, *Escherichia coli* has gained widespread usage for the production of natural products [13]. Significant progress has been made in the synthesis of functional sugars, such as human lactooligosaccharides and hyaluronic acid, in *E. coli*, with some processes even reaching industrial-scale production [14]. However, there have been relatively few reports on the synthetic biotechnology-based production of AOs. Several studies, including those by Kamst et al. [15], Zhang et al. [16], Leppyanen et al. [17] and Geremia et al. [18] have expressed the N-acetylglucosamine transferase gene *nodC* from various bacteria in *E. coli*. Moreover, the product identification methods employed in these studies either lacked quantitative analysis and provided only approximate quantification ranges, or failed to quantify the products based on their specific degrees of polymerization. Cottaz et al. co-expressed the *nodC* gene from rhizobial bacteria and the truncated chitinase gene *chiA* in *E. coli*, successfully synthesizing CHOS with exceptional degrees of polymerization [19]. However, the quantitative detection technique employed, thin-layer chromatography, was found to be inaccurate. Liu et al. reported the use of combinatorial pathway engineering to achieve *de novo* production of CHOS in *Bacillus subtilis*, with the production reaching 4.82 g/L in a 3-L fed-batch bioreactor, representing the highest reported level thus far [20]. However, *B. subtilis* has the drawback of producing spores with exceptional stress resistance under nutrient-deprived conditions, leading to potential environmental pollution and challenging control of the industrial fermentation process [21]. Therefore, there is a significant need to develop and improve the synthesis process of AOs through the application of synthetic biotechnology.

In order to achieve the biosynthesis of AOs with well-defined structures in *E. coli* through metabolic engineering, this study conducted a

Table 1
Strains and plasmids were used in this study.

Strains or plasmids	Description	Source
strains		
BL27	MG1655 F-*lambda-ilvG-rfb*-50 *rph-1* Δ*hsdR* Δ*ampC* lacZ::T7	Prof. Quan [22]
CHO1	*E. coli* BL27 Δ*chiA*	this study
CHO2	*E. coli* BL27 Δ*nagZ* Δ*chiA*	this study
CH1	CHO2 harboring plasmids pET28a-*nodCG*	this study
CH2	CHO2 harboring plasmids pET28a-*nodCL*	this study
CH3	CHO2 harboring plasmids pET28a-*nodCP*	this study
CH4	CHO2 harboring plasmids pET28a-*nodCL*, Δ*nagB*	this study
CH5	CHO2 harboring plasmids pTrc99a-*nodCL*, Δ*nagB*	this study
H121	CHO2, Δ*nagB*, *fliR*::*nodCL*	this study
H1211	CHO2, Δ*nagB*, *fliR*::*nodCL*, *flgA*::*nodCL*	this study
H1212	CHO2, Δ*nagB*, *fliR*::*nodCL*, *flgA*::*nodCL*, *arsB*::*nodCL*	this study
H1213	CHO2, Δ*nagB*, *fliR*::*nodCL*, *flgA*::*nodCL*, *arsB*::*nodCL*, *flgG*:: *nahK*	this study
plasmids		
pREDCas9	Spe^R, Cas9 and λ Red recombinase expression vector	Prof. Wu [23]
pGRB	Amp^R, gRNA expression vector	Prof. Wu [23]
pET28a	Kan^R, T7 promoters	lab stock
pTrc99a	Amp^R, Trc promoters	lab stock
pET28a-*nodCG*	pET28a with gene *nodCG*	this study
pET28a-*nodCL*	pET28a with gene *nodCL*	this study
pET28a-*nodCP*	pET28a with gene *nodCP*	this study
pET28a-*nahK*	pET28a with gene *nahK*	this study
pTrc99a-*nodCL*	pTrc99a with gene *nodCL*	this study
pGRB-*chiA*	pGRB containing sgRNA targeting to *chiA*	this study
pGRB-*nagZ*	pGRB containing sgRNA targeting to *nagZ*	this study
pGRB-*nagB*	pGRB containing sgRNA targeting to *nagB*	this study
pGRB-*fliR*	pGRB containing sgRNA targeting to *fliR*	this study
pGRB-flgA	pGRB containing sgRNA targeting to *flgA*	this study
pGRB-flgG	pGRB containing sgRNA targeting to *flgG*	this study
pGRB-arsB	pGRB containing sgRNA targeting to *arsB*	this study

series of experiments. Firstly, we screened the glycosyltransferase NodC, which led to the accumulation of CHOS with the DP below 6. Next, we investigated the impact of blocking the NAG degradation pathway, the influence of integrating and varying copy numbers of the glycosyltransferase gene in the genomic context, the function of the UDP-N-acetylglucosamine (UDP-GlcNAc) heterologous *salvage* synthesis pathway, and the fermentation conditions on CHOS synthesis. Following the successful microbial production of CHOS, the process was scaled up in a 5-L bioreactor, and the final titers and composition of CHOS were analyzed and evaluated.

2. Material and methods

2.1. Strains, plasmids, media and culture conditions

The strain *E. coli* BL27 was chosen as the starting strain and subjected to genetic modifications to enhance the production of CHOS. Detailed information regarding the relevant plasmids and strains can be found in Table 1. All microorganisms were cultured at a temperature of 37 °C and an agitation rate of 220 rpm in Luria-Bertani (LB) broth, comprising 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. For seed culture and strain activation, LB agar plates were also utilized. When necessary, specific antibiotics such as ampicillin (100 μg/mL), kanamycin (50 μg/mL), or spectinomycin (100 μg/mL) were incorporated into the media to facilitate plasmid selection or gene editing processes.

For shake-flask cultivation, a single colony was selected from the agar plate and transferred directly into a 10 mL test tube containing 2 mL of LB medium. The test tube was then incubated at 37 °C with agitation at 200 rpm in a rotary shaker overnight to allow for growth. The seed culture was subsequently transferred into a 500 mL shake-flask containing 100 mL of the cultivation medium. Glucose-defined medium A is composed of 20 g/L glucose, 13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 1.4 g/L $MgSO_4{\cdot}7H_2O$, 4.5 mg/L thiamine, and 10 mL/L of a trace element solution (10 g/L $FeSO_4{\cdot}7H_2O$, 2.2 g/L $ZnSO_4{\cdot}7H_2O$, 1.0 g/L $CuSO_4{\cdot}5H_2O$, 0.38 g/L $MnSO_4{\cdot}H_2O$, 0.02 g/L $Na_2B_4O_7{\cdot}10H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24}$, and 2.0 g/L $CaCl_2$). Glucose (20 g/L) in medium A was replaced with 20 g/L glycerol in medium B. Medium C:11.8 g/L tryptone, 23.6 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 20 g/L glycerol. The cultivation process was conducted at a temperature of 37 °C with agitation at 200 rpm for approximately 3–5 h. When the optical density at 600 nm (OD_{600}) reached a range of 0.6–0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) and NAG were added to the culture at final concentrations of 0.5 mM and 10 g/L, respectively. Subsequently, the cultivation was continued for 72 h at 37 °C with agitation at 200 rpm.

2.2. Chemicals and reagents

The standards of CHOS and UDP-GlcNAc were obtained from Zhen Zhun (Shanghai, China) and Carbosynth (Berkshire, UK), respectively. Molecular biology kits and reagents were obtained from Takara (Dalian, China) or Vazyme (Nanjing, China). NAG was obtained from Sinopharm (Shanghai, China). Silicone defoamer WH-II was purchased from Changzhou Jiangdong Auxiliary Co., Ltd. (Changzhou, China). Other chemical reagents were analytically pure.

2.3. Plasmid construction

The ClonExpress II One-step Cloning Kit (Vazyme Biotech, Nanjing, China) was employed to construct the target plasmid. Specifically, the target gene fragment was inserted into the pET28a (or pTrc99a) vector at the *Bam*HI/*Sal*I (or *Eco*RI/*Bam*HI) sites using the aforementioned kit. This resulted in the generation of various plasmids, namely pET28a-*nodCG*, pET28a-*nodCL*, pET28a-*nodCP*, pET28a-*nahK*, and pTrc99a-*nodCL*. The genes *nodCG*, *nodCL*, *nodCP*, and *nahK* were synthesized by Genewiz (Suzhou, China). The specific primers employed in this study can be found in Supplementary data.

2.4. Genome integration and gene deletion

Gene knockin and knockout strains were conducted using the CRISPR/Cas9 system [24]. To construct the sgRNA integron expression plasmid, the N20 sequence for the integrated and missing lociwas designed using the CHOPCHOP website, based on the template plasmid pGRB [25]. For knocking out the *nodCL* gene in the BL27 locus of *E. coli*, the homologous upstream and downstream arms of the integrated locus were obtained from the BL27 locus of *E. coli*. Subsequently, the reading frames of the target gene P_{T7}-*nodCL* were amplified from pET28a-*nodCL* using primers *fliR-nodCL*-F/R. To integrate the P_{T7}-*nodCL* gene into the genome of *E. coli* BL27, knockout genes and homologous fragments of knockout genes were assembled through fusion PCR. Similarly, the P_{trc}-*nodCL* and P_{T7}-*nahK* genes were integrated into the *E. coli* genome using the same procedure.

2.5. Fed-batch fermentation

The fed-batch culture was conducted using a 5-L bioreactor (Bailun, Shanghai, China). The working volume of the bioreactor was 2.5 L, with medium A containing 3 g/L yeast extract. The inoculum comprised 20 % (v/v) of the working volume. Throughout the fermentation process, the temperature was maintained at 37 °C, and the pH was controlled at 6.8 using ammonia solution. Once the OD_{600} value reached 13, IPTG and NAG were added at concentrations of 0.5 mM and 2 g/L, respectively. A glucose solution with a concentration of 800 g/L and $MgSO_4{\cdot}7H_2O$ at 20 g/L was continuously fed into the fermentation broth to supplement the

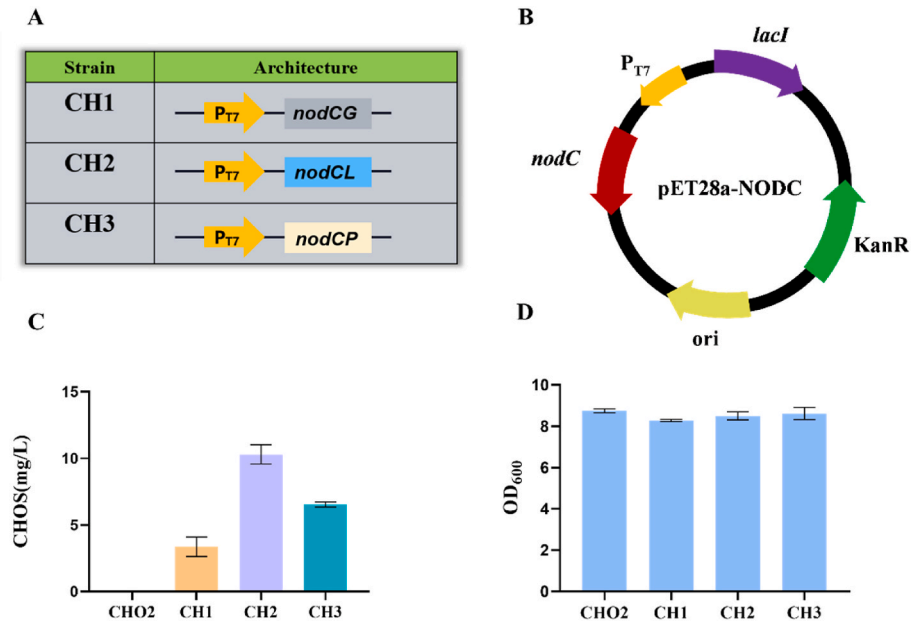

Fig. 2. Production of CHOS *via* expressing *nodC* from different sources in recombinant *E. coli*. (A) Construction of recombinant *E. coli* containing NodC from three different sources. (B) Map of the plasmid pET28a-*nodC* which contains a constitutive promoter P_{T7} and a copy of codon-optimized *nodC*. (C) Effects of various sources of *nodC* on the titer of CHOS. (D) Effects of various sources of *nodC* on cell growth. All data were the average of triplicates with standard deviations.

carbon source, regulated by a dissolved oxygen (DO) mode statistical mode.

2.6. Analytic methods

Biomass was determinied by OD_{600} using a spectrophotometer. The concentrations of glucose and NAG in the fermentation broth were measured with a Shimadzu high-performance liquid chromatography (HPLC) system (LC-20AT) equipped with an Asahipak NH2P-50 column (Shodex, Tokyo, Japan) and an evaporative light scattering detector (ELSD-LTII). The mobile phase was 70 % acetonitrile with a flow rate of 0.8 mL/min and the column temperature was maintained at 40 °C. Qualitative and quantitative analysis of CHOS was conducted using a UPLC system (LC-30A, Shimadzu, Japan) coupled with a Q-Exactive mass spectrometer (Thermo Fisher, USA). The UPLC method employed in this study was consistent with the approach described in a previous study [20]. To determine the content of UDP-GlcNAc in the fermentation broth, the following UPLC system configuration was utilized: Chromatographic separation was carried out using an Acquity BEH Amide column (100 mm × 2.1 mm, 1.7 μm, Waters, USA) maintained at 45 °C. Mobile phase A consisted of a 50 mmol/L ammonium formate solution adjusted to a pH of 4.5 with formic acid, while mobile phase B consisted of 100 % acetonitrile. The gradient program for the mobile phase was as follows: 0–1.8 min, 20 % A; 1.8–14 min, 20 % A to 50 % A; 14–16 min, 50 % A to 55 % A; 16–17 min, 55 % A to 20 % A; 17–20 min, the composition was maintained at 20 % A. The flow rate of the mobile phase was set to 0.3 mL/min, and each sample was injected at a volume of 10 μL.

The mass spectrometry parameters were set as follows: the scanning range was 80.0–1200.0 *m/z*. The ion source operated in positive ion mode with sheath gas voltage at 40 arb, auxiliary gas voltage at 10 arb, capillary temperature at 350 °C, ion spray voltage at 3.5 kV, and resolution set to 35000. A full scan of CHOS in positive ion mode was conducted to examine their ion abundance in various ionization modes, such as $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$. The ions with the highest abundance were selected as the quantitative ions. All experiments were performed in triplicate to ensure reproducibility.

3. Results and discussion

3.1. Construction of the recombinant E. coli for the synthesis of CHOS

Fig. 1B illustrates the pathway for CHOS synthesis in *E. coli* using glucose as the carbon source. Glucose is first converted to glucose-6-phosphate by the action of glucokinase. Subsequently, four enzymes catalyze the conversion of glucose-6-phosphate to UDP-GlcNAc. UDP-GlcNAc serves as the glycosyl donor for CHOS synthesis in the presence of the exogenous glycosyltransferase NodC. It should be noted that *E. coli* has the capability to degrade CHOS under certain conditions. The endogenous protein ChiA in *E. coli* can break down CHOS5 into CHOS3 and CHOS2. Though the expression level of ChiA is relatively low when the carbon source is abundant, the expression of ChiA can be induced in carbon-deficient conditions to enable *E. coli* to utilize chitosan or CHOS as the sole carbon source. On the other hand, NagZ is a specific protein that hydrolyzes NAG linked with β-1,4-glycosidic bonds, allowing chitosan or CHOS to be broken down into monosaccharides for cellular utilization [26]. To prevent the degradation of synthesized CHOS, in this study, the CHO2 strain was generated first by knocking out the chitinase gene (*chiA*) and Nacetylglucosidase gene (*nagZ*) in the chassis cells.

To enable *E. coli* to utilize endogenous UDP-GlcNAc for CHOS synthesis, the introduction of exogenous glycosyltransferase NodC is necessary. However, the activity of NodC can vary depending on its source. Therefore, three different NodC-encoding recombinant plasmids, namely pET28a-*nodCG*, pET28a-*nodCL*, and pET28a-*nodCP*, which expresses NodCG from *Azorhizobium caulinodans* [18], NodCL from *Mesorhizobium Loti* [16], and NodCP from *Rhezobium leguminosarum bv. viciae* [15], respectively, were constructed. Each plasmid was individually introduced into the CHO2 strain to generate strains CH1, CH2, and CH3.

As depicted in Fig. 2D, the introduction of three different glycosyltransferases, NodC, had no discernible impact on the growth rate of *E. coli*. Fig. 2C demonstrated that *E. coli* strain CHO2 was unable to synthesize CHOS, while strains CH1, CH2, and CH3 expressing different NodC were all capable of producing CHOS. This indicated that *E. coli* lacked endogenous glycosyltransferases for CHOS synthesis and it was necessary to introduce exogenous glycosyltransferases in the engineered *E. coli* cell factory for CHOS production.

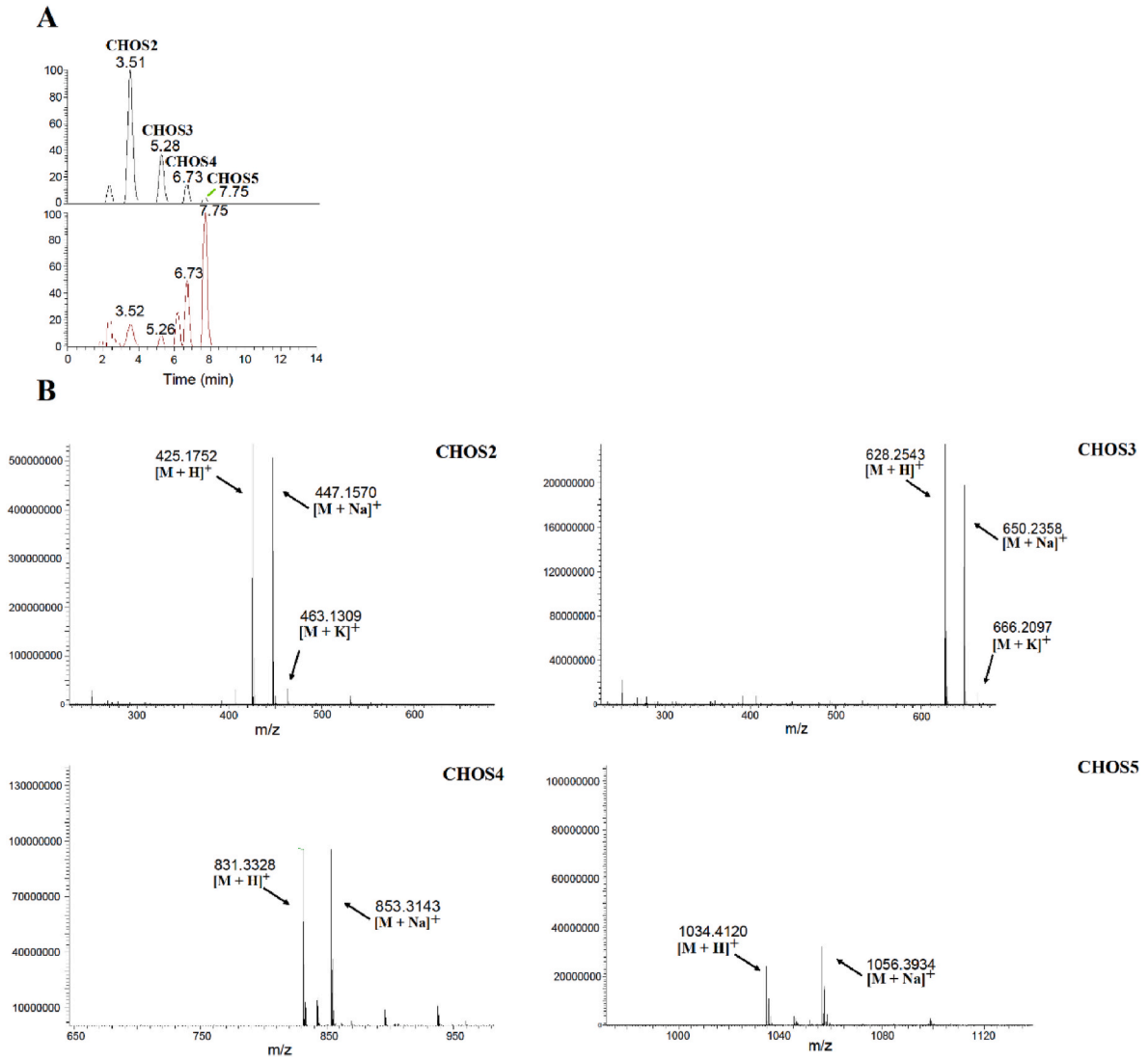

Fig. 3. Identification and quantitation of product CHOS. (A) Chromatograms of CHOS2, CHOS3, CHOS4 and CHOS5. The black color above represents the standard samples, while the red below represents the strain CH2 fermentation broth sample. (B) Mass spectra of CHOS2, CHOS3, CHOS4, and CHOS5 in the strain CH2.

Among these strains, CH2 exhibited the highest CHOS production (10.6 mg/L), while CH1 produced the least (3.4 mg/L). This observation was consistent with previous research reports [16]. Meixi Ling et al. examined six different exogenous COS synthase candidates from *Sinorhizobium meliloti*, *Bradyrhizobium* sp. *WSM3983*, *Neorhizobium galegae bv. officinalis HAMBI 1141, Rhizobium leguminosarum bv. trifolii WSM2012*, *Paraburkholderia terricola LMG* and *Mesorhizobium loti*, and found the synthase from *M. loti* had the highest CHOS titer [20]. Dawei Zhang et al. constructed recombinant *E. coli* DCL3 and DCM3 expressing NodCL and NodCM from *M. loti* and *S. meliloti*, respectively. Culture results showed that the cell density and oligosaccharide synthesis efficiency of recombinant bacteria DCL3 were significantly higher than those of recombinant bacteria DCM3, which reflected the excellent oligosaccharide production capacity of NodCL from *M. Loti* [16]. As both the above reports and our study highlighted the superior CHOS production capacity of NodCL from *M. loti*, subsequent investigations focused on further exploring the potential of NodCL.

In order to determine the DP and structural characteristics of the synthesized CHOS product in CH2, qualitative and quantitative analysis of CHOS was performed using liquid chromatography mass spectrometry (LC-MS). As shown in Fig. 3, by analyzing the peak retention time in the chromatogram and the corresponding mass spectrometry data with mass tolerance of less than 5 ppm, the product was identified as CHOS with a DP ranging from 2 to 5.

3.2. The effect of blocking the GlcNAc degradation pathway on the synthesis of CHOS

E. coli exhibits a preference for NAG as a carbon source, similar to glucose. The enzyme glucosamine-6-phosphate deaminase (NagB) plays a crucial role in enabling *E. coli* to utilize NAG as its sole carbon source because NagB catalyzes the conversion of glucosamine-6-phosphate into fructose-6-phosphate which can directly enter the glycolysis pathway. To prevent NAG from entering the glycolysis pathway and improve its utilization for the synthesis of UDP-GlcNAc, strain CH4 was constructed by knocking out the *nagB* gene in strain CH2 (Fig. 4A).

Shake flask fermentation results of strains CH2 and CH4 showed that the knockout of the *nagB* gene did not affect the growth of *E. coli* in 72 h (Fig. 4B). Strain CH2, without the *nagB* gene knockout, consumed 57 % of NAG with a conversion rate of 0.002 g/g. In contrast, strain CH4, with the *nagB* gene knockout, consumed only 25 % of NAG, achieving a NAG conversion rate of 0.008 g/g, four times higher than that of the control strain CH2. Furthermore, strain CH4 exhibited a final CHOS accumulation of 20.6 mg/L, double that of strain CH2 (10.3 mg/L) (Fig. 4C).

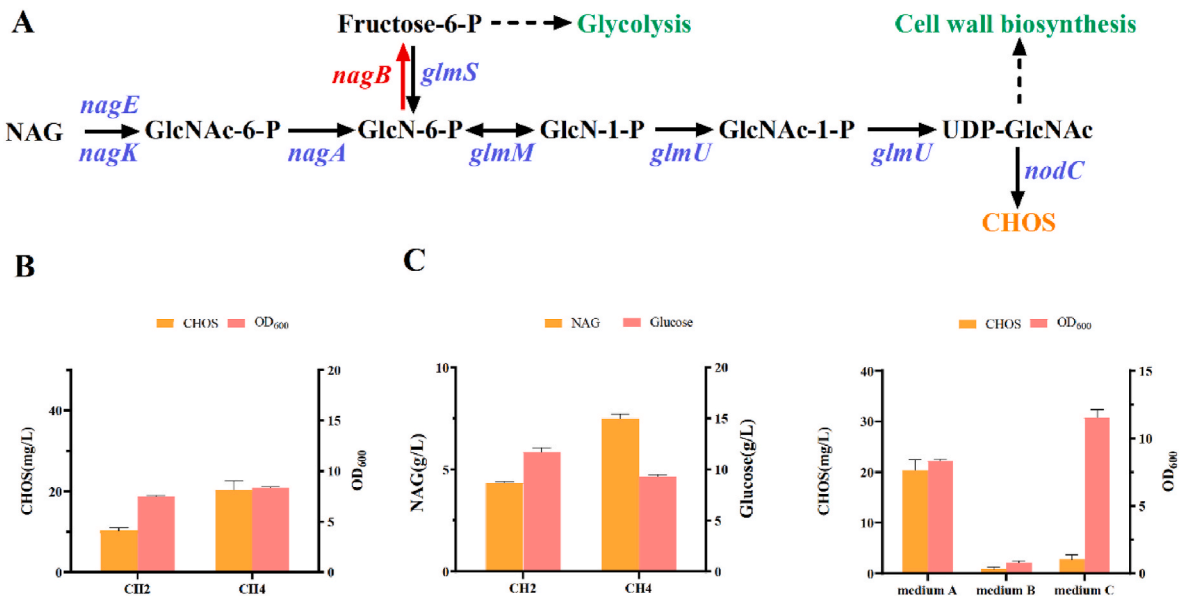

Fig. 4. The effect of knocking out *nagB* gene on the synthesis of CHOS. (A) Pathway diagram of *nagB* gene knockout strain CH4. (B) CHOS production and cell growth of different recombinant strains. (C) The surpluses of carbon source NAG and glucose of different recombinant strains. (D) CHOS production and cell growth of different media. All data were the average of triplicates with standard deviations.

These results demonstrated that knocking out the *nagB* gene reduced NAG consumption, enhanced the conversion rate of NAG, and increased CHOS production.

3.3. The effect of carbon source on CHOS synthesis

To investigate the effect of different carbon source media on CHOS synthesis, strain CH4 was fermented using various media in shake flasks. Specifically, medium A with glucose as the sole carbon source, medium B with glycerol as the sole carbon source and compound medium C with glycerol as the carbon source were examined. The results showed that the maximum CHOS titer of medium A was 20.4 mg/L. However, medium B led to a significant decrease in cell growth (OD_{600}) and a very low CHOS titer (Fig. 4D). The above results indicated that in medium A, even after the addition of NAG at OD_{600} between 0.6 and 0.8, glucose was still used as the carbon source. In medium B, compared to glycerol, NAG was more easily utilized and better absorbed as a carbon source, resulting in the Crabtree effect, which suppressed cell growth and inhibited CHOS titers. Therefore, medium B was deemed unsuitable for CHOS production. On the other hand, the addition of a variety of nutrients to compound medium C promoted rapid strain growth, with OD_{600} exceeding the levels of medium A and B, while also increasing the

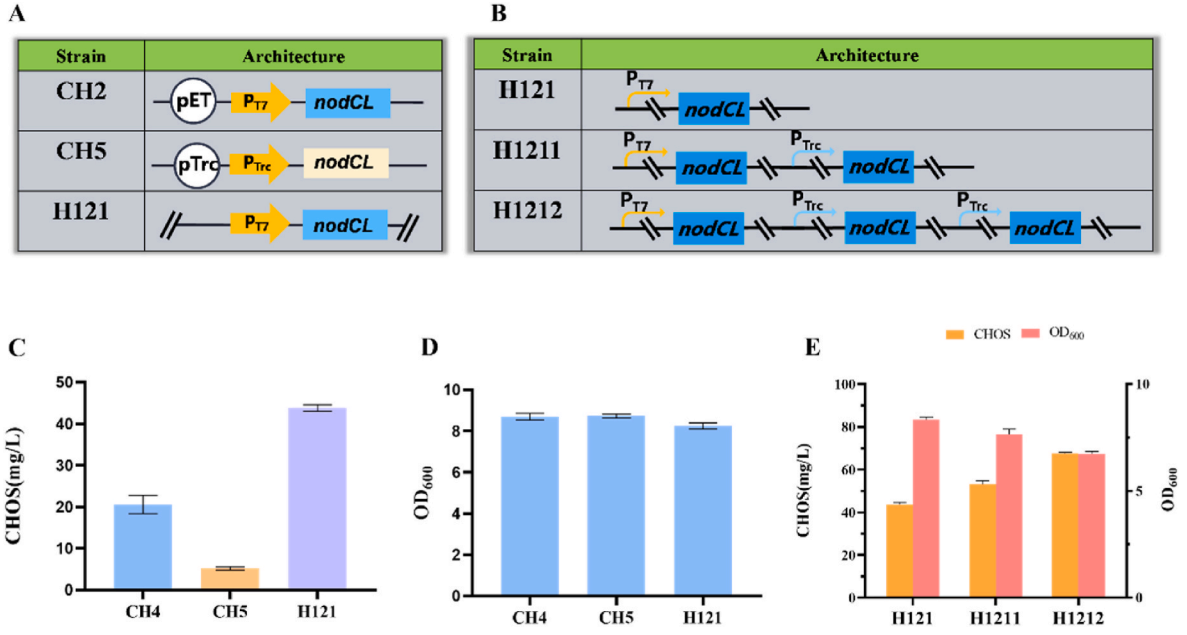

Fig. 5. The effect of different expression systems on the synthesis of CHOS. (A) Expression systems for the recombinant strains of different expression levels of *nodCL*. (B) Construction of the strains with different copy numbers of *nodCL*. (C) CHOS production of recombinant strains CH4, CH5, and H121. (D) Cell growth of recombinant strains CH4, CH5, and H121. (E) CHOS production and cell growth of recombinant strains H121, H1211, and H1212. All data were the average of triplicates with standard deviations.

protein expression rate. However, excessively fast protein translation hindered the correct folding of a large number of recombinases, resulting in reduced active recombinase levels and a decline in CHOS production catalyzed by the recombinase, resulting in the highest OD_{600} but no corresponding increase in CHOS titer. Hence, medium C was also considered unsuitable for CHOS production. Considering its excellent performance, medium A was selected for further investigation, and the specific components of the medium are described in Material and methods.

3.4. The effect of NodC expression level and stability on the synthesis of CHOS

Considering the critical role of glycosyltransferase NodC in CHOS synthesis, the expression level, activity and stability of NodC may have a great effect on CHOS synthesis in recombinant *E. coli*. First, the *nagB* gene in strain CHO2 was deleted by CRISPR-Cas9 system and strain CHO3 was constructed. Next, CH5 was generated through plasmid pTrc99a-*nodCL* transformation into strain CHO3 and strain H121 was obtained by integrating the expression cassette of plasmid pET28a-*nodCL* into the genome of strain CHO3. Then, strains CH4, CH5, and H121 were cultured and their CHOS productions were evaluated. As shown in Fig. 5C, compared with strain CH5 (5.1 mg/L), strain CH4 produced much more CHOS (20.6 mg/L), about 4.04-fold. The result indicated that T7 promoter was stronger than trc promoter and T7 promoter was more suitable for NodC expression. In contrast to strain CH4 harboring plasmid pET28a-*nodCL*, strain H121 with the expression cassette of plasmid pET28a-*nodCL* integrated into genome produced 1.13 times more CHOS, reaching 43.8 mg/L, which verified that the *NodC* gene integration expression was more stable.

To investigate the impact of copy numbers of genes on CHOS synthesis, strain H1211, containing two-copy *nodCL* expression cassettes inserted at the fliR and flgA sites, and strain H1212, with three-copy *nodCL* expression cassettes inserted at the fliR, flgA and arsB site, were constructed (Fig. 5B). As displayed in Fig. 5E, strain H1211 showed a 21.2 % increase in CHOS production compared to H121, reaching 53.1 mg/L. Strain H1212 exhibited another 27.3 % increase in CHOS production compared to H1211, reaching 67.6 mg/L. Whereas, the cell amounts in H121, H1211 and H1212 decreased gradually. The results indicated that increasing the expression of *NodC* gene through adding gene copy-number can indeed enhance the yield of the target product CHOS, while adding gene copy-number may also cause cell growth burden.

Increasing the copy numbers of the expression cassette and then integrating the multi-copy gene into the microbial genome was currently one of the conventional tools and more widely used solutions for obtaining improved gene expression and thus increased production of the desired substance. Not only was this method utilized in this study, but it was also good in the synthesis of other bioactive substances. Single-copy integration of heterologous pathway genes limited the production of flavonoids. The multi-copy integration of flavonoid pathway genes effectively improved (2S)-naringenin production in *Saccharomyces cerevisiae*. Higher copy numbers of the (2S)-naringenin metabolic pathway genes were associated with greater essential genes' transcription, and the efficiency of naringenin production was higher [27]. Through constructing a series of vectors harboring different copy numbers of Rhizopus oryzae lipase (ROL) gene cassettes transforming the plasmids into *Pichia pastoris* to generate a series of strains with specific copy numbers of ROL and screening, an optimized gene-dosage recombinant strain harboring five copies of ROL with the highest production was obtained, which was 8-fold higher than that of the strain harboring one copy [28]. Also in *Pichia Pastoris*, to optimize the expression level of the recombinant ergopeptine hydrolase ErgA, Julia Panholzl et al. constructed plasmids with one, two, and three cassettes of ergopeptine hydrolase under control of the same promoter and found that two copies of the ergA gave higher ErgA yield than one copy, but a third copy of ergA showed little or no further increase [29]. Similar experiments were also used in penicillin-producing industrial strains. For *Penicillium chrysogenum*, the fungal producer of the beta-lactam antibiotic penicillin, many production strains carried multiple copies of the penicillin biosynthesis gene cluster. This discovery led to the generally accepted view that high penicillin titers are the result of multiple copies of penicillin genes. But Sandra Ziemons et al. showed that the enhanced penicillin titer did not strictly depend on the copy number of the cluster, complex regulatory mechanisms also played a crucial role in the synthesis of bioactivities in production strains [30].

In this study, each additional copy of the *nodCL* expression cassette resulted in an approximate 20 % improvement in CHOS production capacity. Strain H1212 achieved a 54.3 % increase in CHOS production compared to H121 (Fig. 5E). Consequently, subsequent experiments were conducted using strain H1212.

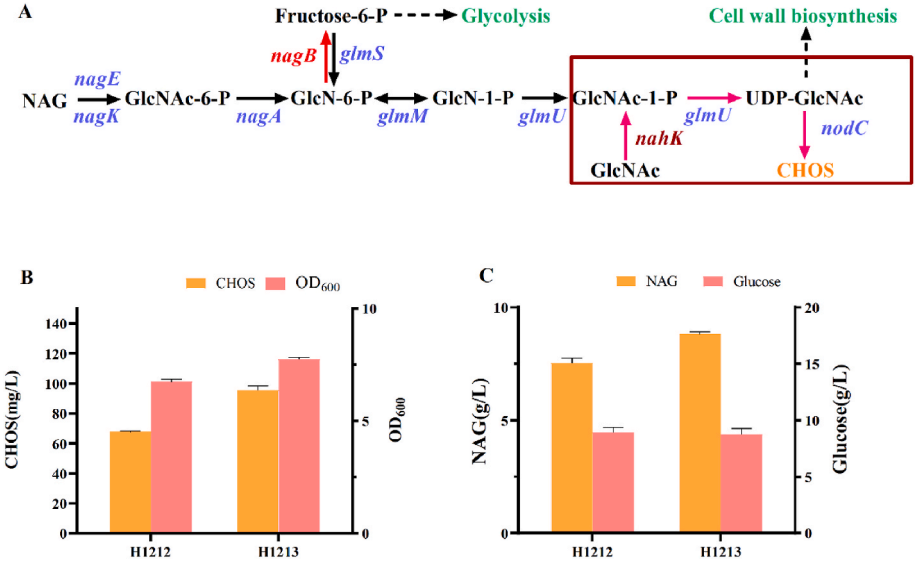

Fig. 6. The effect of introducing heterologous *salvage* pathway on the synthesis of CHOS. (A) Pathway diagram of *nahK* gene knockin strain H1213. (B) CHOS production and cell growth of recombinant strains H1212 and H1213. (C) The surpluses of carbon source NAG and glucose. All data were the average of triplicates with standard deviations.

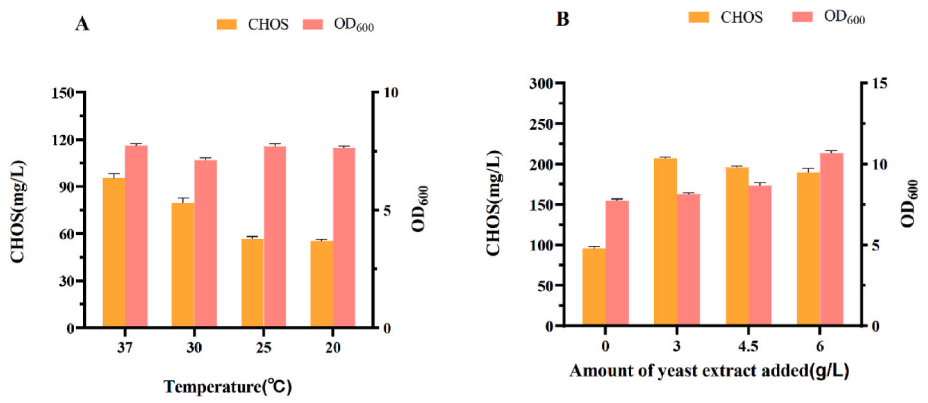

Fig. 7. The effect of different fermentation conditions on the synthesis of CHOS. (A) The cell growth and CHOS production of H1213 after fermentation at different temperatures. (B) The cell growth and CHOS production of H1213 after fermentation at different additions of yeast extract. All data were the average of triplicates with standard deviations.

3.5. The effect of introducing UDP-GlcNAc salvage pathway on the synthesis of CHOS

UDP-GlcNAc is an important nucleotide sugar precursor involved in the synthesis of biological macromolecules, such as chitin and mannoglycoprotein in fungi, peptidoglycan and lipopolysaccharide in bacteria, and lipopolysaccharide in animal cells. In the process of CHOS synthesis, UDP-GlcNAc is added to monosaccharide NAG to synthesize CHOS2 or the growing CHOS chain to increase the DPs of CHOS. The higher the DPs, the more UDP-GlcNAc is needed. Theoretically, the synthesis of one molecule CHOS2 requires one molecule of UDP-GlcNAc, while the synthesis of one molecule CHOS5 requires four molecules of UDP-GlcNAc. Therefore, the supply concentration of glycosyl donor UDP-GlcNAc affects the DP of the sugar chain and the amount of CHOS products to some extent. It has been reported that low UDP-GlcNAc concentrations led to an increased proportion of oligosaccharide products with low polymerization in yeast [16,31]. Likewise, the shorter oligomers in *E. coli* could result from lower intracellular UDP-GlcNAc concentrations.

The supply concentration of UDP-GlcNAc may also affect the synthesis amount of CHOS. Sanmain et al. discussed the relationship between the synthesis of intracellular CHOS and the limited supply of UDP-GlcNAc. They found the low growth rate of recombinant *E. coli* under anabolic culture ensured a higher concentration of UDP-GlcNAc and the synthesis of CHOS proceeded smoothly by NodC. However, if the growth rate was too fast, the lysogenic phenomenon would be caused, because of the insufficient supply of UDP-GlcNAc [32]. Though the metabolic pathway leading to UDP-GlcNAc is a native pathway in *E. coli*, the productivity is extremely low. Increasing the availability of UDP-GlcNAc can enhance the synthesis of oligomers composed of GlcNAc. It has been reported that overexpressing one or several genes for GlcNAc synthesis—*glmS*, *glmM*, and *glmU* [33], the UDP-GlcNAc pool in *Lactobacillus casei* increased by a factor of four [34], the production of hyaluronan, a copolymer of glucuronic acid and GlcNAc, increased nearly 3-fold in *E. coli* [35].

Except for *de novo* synthesis, UDP-GlcNAc can also be synthesized through the *salvage* pathway, in which exogenous NAG is first converted to N-acetylglucosamine-1-phosphate (NAG-1-P) by N-acetylhexosamine 1-kinase (NahK), followed by transformed into UDP-GlcNAc through NAG-1-P by uridyltransferase (GlmU). To verify the feasibility of applying heterologous UDP-GlcNAc *salvage* pathway on CHOS synthesis, the enzyme NahK from *Bifidobacterium longum* subsp. *Longum* [36] was cloned into the plasmid pET28a, resulting in the recombinant plasmid pET28a-*nahK*. Strain H1213 was generated by integrating the gene expression cassette of *nahK* on pET28a-*nahK* into the genome of *E. coli* strain H1212 (Fig. 6A).

The growth and the production of CHOS of strain H1213 and its control strain H1212 were compared. As shown in Fig. 6B, compared to strain H1212, the introduction of the *nahK* gene did not decrease the growth of *E. coli*, instead, the OD_{600} of strain H1213 after 72 h culture increased by 12.3 %, and the CHOS yield of H1213 increased by 41.3 %, reaching 95.5 mg/L. Carbon source consumption analysis suggested the utilization of glucose was similar in strain H1212 and strain H1213, while strain H1213 exhibited a reduced overall consumption of NAG and increased NAG conversion rate compared to H1212 (Fig. 6C). The conversion rate of NAG in strain H1213 was 0.080 g/g, 2.96-fold that of the control strain H1212 (0.027 g/g). To our knowledge, this was the first report to introduce a heterologous UDP-GlcNAc *salvage* pathway for CHOS synthesis. The culture conditions and expression balance among NahK and other CHOS synthesis related proteins would be studied further.

3.6. Optimization of fermentation conditions and batch fermentation with 5-L fed-batch fermentation

To obtain high titers of CHOS, fed-batch cultivation conditions of the optimal CHOS accumulating strain H1213 were employed. The culture temperature affects the cell's growth rate, the soluble expression of recombinant proteins, and the amount of the final product. To obtain the optimal fermentation temperature, strain H1213 was cultured at various temperatures (37 °C, 30 °C, 25 °C, and 20 °C) for 72 h, and then the CHOS yield was evaluated. As depicted in Fig. 7A, lowering the fermentation temperature did not impact the final growth of *E. coli* cells, but decreased CHOS yield, with values of 95.8 mg/L, 79.5 mg/L, 56.7 mg/L, and 55.3 mg/L at 37 °C, 30 °C, 25 °C, and 20 °C, respectively. The highest CHOS yield was obtained at a fermentation temperature of 37 °C. Taking into account the growth level of bacteria and their CHOS production capacity, 37 °C was chosen as the fermentation temperature for further experiments.

Yeast extract is known to promote cell proliferation and maintain metabolic flux equilibrium in cells. A previous study found an increase in intracellular UDP-GlcNAc levels as well as an increased COS production in *E. coli* after the addition of yeast extract to the medium [16]. To find a suitable yeast extract additive amount, in this study, different concentrations of yeast extract (3.0 g/L, 4.5 g/L, and 6.0 g/L) were added to the fermentation medium. As illustrated in Fig. 7B, the addition of yeast extract influenced both cell density (OD_{600}) and CHOS production. Specifically, at yeast extract concentrations of 3.0 g/L, 4.5 g/L, and 6.0 g/L, the corresponding cell densities were 8.1, 8.7, and 10.7, respectively, while CHOS production reached 207.1 mg/L, 195.8 mg/L, and 192.0 mg/L, respectively. The highest CHOS production was achieved with 3.0 g/L yeast extract addition, resulting in a 116.9 % increase compared to the original medium without yeast extract. So, 3.0 g/L yeast extract addition was selected in further studies.

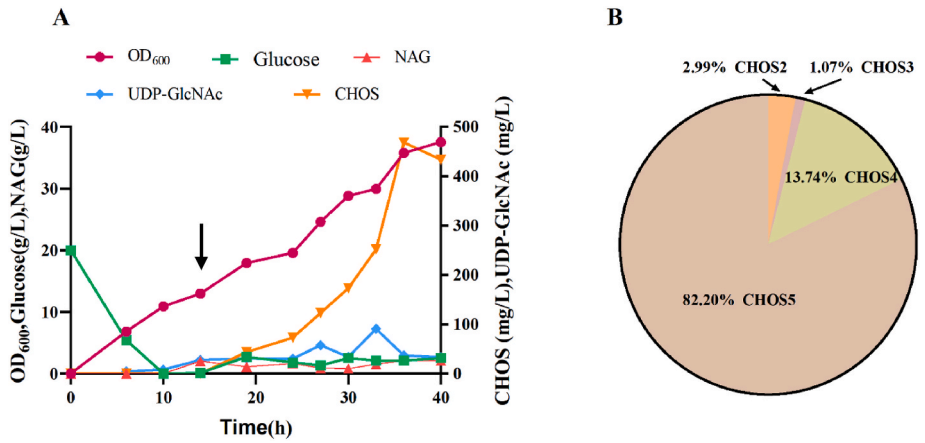

Fig. 8. Scale-up production of CHOS in a 5-L bioreactor. (A) Variation of the cell growth, the surpluses of carbon source NAG and glucose, and the yield of CHOS and UDP-GlcNAc with fermentation time. The black arrow represents the addition of IPTG and substrate NAG. (B) The proportion of CHOS with different DPs in the mixture product.

3.7. Production of CHOS in the 5-L bioreactor

To evaluate the performance of strain H1213 under more industrial production conditions, we performed fed-batch fermentation in a 5-L bioreactor with medium A and intermittent feeding of glucose and NAG. The pH in the fermentation system was maintained at 6.8 by intermittently supplementing 14 % ammonia solution. At 14 h of fermentation, the cell density was 13.0. At this time, IPTG with a final concentration of 0.5 mmol/L was added to induce the expression of the target protein in the fermentation system, while NAG with a final concentration of 2 g/L was added as the substrate. The consumption of NAG was accompanied by the formation of CHOS. During the fermentation, the glucose was intermittently supplemented to the bioreactor to maintain the concentration of no more than 5 g/L to reduce the generation of by-product lactic acid. At 36 h of fermentation, the concentration of CHOS reached 468.6 mg/L. With this process, the highest concentration of UDP-GlcNAc was 91.0 mg/L, the highest level reported in *E. coli* so far, and the highest cell density reached 37.6. As shown in Fig. 8B, the DP range of the synthesized CHOS was between 2 and 5. The specific distribution was as follows: CHOS2: 3.0 %, CHOS3: 1.1 %, CHOS4: 13.7 %, CHOS5: 82.2 %. At present, most of the products obtained by enzymatic degradation are only CHOS2 and CHOS3 [37,38]. This study increased the range of DP of CHOS and expanded the possibility of application of CHOS. The conversion rate of glucose was measured at 0.021 g/g, while the conversion rate of NAG was 0.049 g/g. Comparatively, fed-batch fermentation demonstrated notable improvements in cell density and CHOS production compared to shake flask fermentation methods.

4. Conclusions

In summary, this study constructed a combinatorial *de novo* pathway and *salvage* pathway for producing structurally defined and homogeneous CHOS by remodeling the metabolic network in *E. coli* BL27. We first knocked out the genes of *chiA* and *nagZ* that can degrade CHOS, and constructed recombinant *E. coli* CHO2 for the synthesis of CHOS, and then identified NodCL from *M. loti* as the most suitable exogenous glycosyl transferase for CHOS production. By blocking the GlcNAc degradation pathway, adjusting the copy numbers of nodCL expression cassette, introducing the heterologous *salvage* pathway and optimizing fermentation conditions, CHOS production was increased gradually, reaching 207.1 mg/L in shake-flask cultivation and 468.6 mg/L in a 5-L fed-batch bioreactor. The synthesized CHOS was composed of only NAG, and the main DPs were CHOS5 (82.2 %) and CHOS4 (13.7 %). The successful biosynthesis of well-defined AOs in plasmid-free *E. coli via* metabolic engineering techniques provides a starting point for further engineering and commercial production of CHOS. To our knowledge, this was the first report to introduce a heterologous UDP-GlcNAc *salvage* pathway for CHOS synthesis, and it was currently the highest reported yield in *E. coli*. As a donor of CHOS and various other oligosaccharides, the increase in UDP-GlcNAc production also provided more possibilities for the divergence of synthesis pathways and potential increase in yield of CHOS and other more oligosaccharides. However, the yield of CHOS in *E. coli* was lower than that in *B. subtilis* [20]. The main reason is that the cell wall of *B. subtilis* is thicker, and the concentration of the precursor UDP-GlcNAc of CHOS is higher, which is more conducive to the synthesis of CHOS. Moreover, in this study, the growth state of *E. coli* in the bioreactor did not reach a higher density of fermentation, so there was still considerable room for improvement. For example, optimization of culture conditions (the culture medium, temperature, pH and other factors) adjusting multiple crucial nodes in central carbon and nitrogen metabolism, as well as pyrimidine metabolism synthesis pathways, employing global regulatory transcription engineering strategies can potentially maximize the cellular potential and boost CHOS production.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Supplementary files.

CRediT authorship contribution statement

Jinqi Shi: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Validation, Visualization, Writing – review & editing. **Chen Deng:** Investigation, Project administration. **Chunyue Zhang:** Conceptualization, Resources, Data curation, Writing – review & editing. **Shu Quan:** Methodology, Supervision, Resources. **Liqiang Fan:** Methodology, Writing – review & editing. **Liming Zhao:** Conceptualization, Funding acquisition, Resources, Methodology, Supervision, Writing – review & editing, All authors reviewed the manuscript and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Young Scientists Fund of the National Natural Science Foundation of China (32301214), Shanghai Postdoctoral Excellence Program (2022148) and the 111 Project (B18022).

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.05.011.

References

[1] Qu T, Zhang C, Qin Z, Fan L, Jiang L, Zhao L. A novel GH family 20 β-N-acetylhexosaminidase with both chitosanase and chitinase activity from *Aspergillus oryzae*. Front Mol Biosci 2021;8:684086. https://doi.org/10.3389/fmolb.2021.684086.

[2] Berit BA, Ellinor BH, Anne LN, Morten S, Kjell MV, Vincent GHE. Production of chitooligosaccharides and their potential applications in medicine. Mar Drugs 2010;8:1482–517. https://doi.org/10.3390/md8051482.

[3] Chen G, Wu H, Zhu Y, Wan L, Zhang W, Mu W. Glycosyltransferase from *Bacteroides gallinaceum* is a novel α-1,3-fucosyltransferase that can be used for 3-fucosyllactose production in vivo by metabolically engineered *Escherichia coli*. J Agric Food Chem 2022;70:1934–42. https://doi.org/10.1021/acs.jafc.1c06719.

[4] Sánchez Á, Mengíbar M, Rivera-Rodríguez G, Moerchbacher B, Acosta N, Heras A. The effect of preparation processes on the physicochemical characteristics and antibacterial activity of chitooligosaccharides. Carbohydr Polym 2017;157:251–7. https://doi.org/10.1016/j.carbpol.2016.09.055.

[5] Dai N, Moon M, Zhong J, Won K, Sang H, Se K. Free radical-scavenging activities of low molecular weight chitin oligosaccharides lead to antioxidant effect in live cells. J Food Biochem 2010;34:161–77. https://doi.org/10.1111/j.1745-4514.2009.00321.x.

[6] Himadri TB, Abhik M, Smruti RD, Soubhagyalaxmi J, Lopamudra R. Production of N-acetyl chitooligosaccharide by novel *Streptomyces chilikensis* strain RC1830 and its evaluation for anti-radical, anti-inflammatory, anti-proliferative and cell migration potential. Bioresour Technol Rep 2020;11:00428. https://doi.org/10.1016/j.biteb.2020.100428.

[7] Hu H, Xia H, Zou X, Li X, Zhang Z, Yao X, Yin M, Tian D, Liu H. N-acetyl-chitooligosaccharide attenuates inflammatory responses by suppression of NF-κB signaling, MAPK and NLRP3 inflammasome in macrophages. J Funct Foods 2021;78:104364. https://doi.org/10.1016/j.jff.2021.104364.

[8] Zheng J, Cheng G, Li Q, Jiao S, Feng C, Zhao X, Yin H, Du Y, Liu H. Chitin oligosaccharide modulates gut microbiota and attenuates high-fat-diet-induced metabolic syndrome in mice. Mar Drugs 2018;16:66. https://doi.org/10.3390/md16020066.

[9] Harish-Prashanth KV, Tharanathan RN. Chitin/chitosan: modifications and their unlimited application potential—an overview. Trends Food Sci Technol 2007;18:117–31. https://doi.org/10.1016/j.tifs.2006.10.022.

[10] Jeon YJ, Shahidi F, Kim SK. Preparation of chitin and chitosan oligomers and their applications in physiological functional foods. Food Rev Int 2000;16:159–76. https://doi.org/10.1081/FRI-100100286.

[11] Hou F, He L, Ma X, Wang D, Ding T, Ye X, Liu D. Ultrasound enhanced the binding ability of chitinase onto chitin: from an AFM insight. Ultrason Sonochem 2020;67:105117. https://doi.org/10.1016/j.ultsonch.2020.105117.

[12] Xie X, Fu X, Yan X, Peng W, Kang L. A broad-specificity chitinase from *Penicillium oxalicum* k10 exhibits antifungal activity and biodegradation properties of chitin,". Mar Drugs 2021;19:35. https://doi.org/10.3390/md19070356.

[13] Wang C, Pfleger BF, Kim S. Reassessing *Escherichia coli* as a cell factory for biofuel production. Curr Opin Biotechnol 2017;45:92–103. https://doi.org/10.1016/j.copbio.2017.02.010.

[14] Chen Y, Zhu Y, Wang H, Chen R, Liu Y, Zhang W, Mu W. *De novo* biosynthesis of 2′-fucosyllactose in a metabolically engineered *Escherichia coli* using a novel α1,2-fucosyltransferase from *Azospirillum lipoferum*. Bioresour Technol 2023;374:128818. https://doi.org/10.1016/j.biortech.2023.128818.

[15] Kamst E, Drift KM, Thomas-Oates JE, Lugtenberg BJ, Spaink HP. Mass spectrometric analysis of chitin oligosaccharides produced by *Rhizobium* NodC protein in *Escherichia coli*. J Bacteriol 1995;177:6282–5. https://doi.org/10.1128/jb.177.21.6282-6285.1995.

[16] Zhang D, Wang PG, Qi Q. A two-step fermentation process for efficient production of penta-N-acetyl-chitopentaose in recombinant *Escherichia coli*. B Biotechnol Lett 2007;29:1729–33. https://doi.org/10.1007/s10529-007-9462-y.

[17] Leppyanen IV, Artamonova TO, Lopatin SA, Varlamov VP, Tikhonovich IA, Dolgikh EA. Biosynthesis of hexa- and pentameric chitooligosaccharides using N-acetyl-glucoseaminyl transferase from rhizobial bacteria. Russ J Genet Appl Res 2014;4:68–381. https://doi.org/10.1134/S2079059714050098.

[18] Geremia RA, Mergaert P, Geelen D, Van-Montagu M, Holsters M. The NodC protein of Azorhizobium caulinodans is an N-acetylglucosaminyltransferase. Proc Natl Acad Sci 1994;91:2669–73. https://doi.org/10.1073/pnas.91.7.2669.

[19] Cottaz S, Samain E. Genetic engineering of *Escherichia coli* for the production of NI, NII-diacetylchitobiose (chitinbiose) and its utilization as a primer for the synthesis of complex carbohydrates. Metab Eng 2005;7:311–7. https://doi.org/10.1016/j.ymben.2005.05.004.

[20] Ling M, Wu Y, Tian R, Liu Y, Yu W, Tao G, Lv X, Li J, Du G, Amaro RL, Liu L. Combinatorial pathway engineering of *Bacillus subtilis* for production of structurally defined and homogeneous chitooligosaccharides. Metab Eng 2022;70:55–66. https://doi.org/10.1016/j.ymben.2022.01.008.

[21] Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 2006;101:514–25. https://doi.org/10.1111/j.1365-2672.2005.02736.x.

[22] Lai N, Luo Y, Fei P, Hu P, Wu H. One stone two birds: biosynthesis of 3-hydroxypropionic acid from CO(2) and syngas-derived acetic acid in *Escherichia coli*. Synth Syst Biotechnol 2021;6:144–52. https://doi.org/10.1016/j.synbio.2021.06.003.

[23] Li Y, Lin Z, Huang C, Zhang Y, Wang Z, Tang Y, Chen T, Zhao X. Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 meditated genome editing,". Metab Eng 2015;31:13–21. https://doi.org/10.1016/j.ymben.2015.06.006.

[24] Wu HY, Li YJ, Ma Q, Li Q, Jia ZF, Yang B, Xu QY, Fan XG, Zhang CL, Chen N, Xie XX. Metabolic engineering of *Escherichia coli* for high-yield uridine production. Metab Eng 2018;49:248–56. https://doi.org/10.1016/j.ymben.2018.09.001.

[25] Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res 2014;42:W401–7. https://doi.org/10.1093/nar/gku410.

[26] Walter A, Friz S, Mayer C. Chitin, chitin oligosaccharide, and chitin disaccharide metabolism of *Escherichia coli* revisited: Reassignment of the roles of ChiA, ChbR, ChbF, and ChbG. Microb Physiol 2021;31:178–94. https://doi.org/10.1159/000515178.

[27] Li H, GaoZhang S, Zeng W, Zhou J. Effects of metabolic pathway gene copy numbers on the biosynthesis of (2S)-naringenin in *Saccharomyces cerevisiae*. J Biotechnol 2021;325:119–27. https://doi.org/10.1016/j.jbiotec.2020.11.009.

[28] Jiao L, Zhou Q, Su Z, Xu L, Yan Y. High-level extracellular production of *Rhizopus oryzae* lipase in *Pichia pastoris* via a strategy combining optimization of gene-copy number with co-expression of ERAD-related proteins. Protein Expression Purif 2018;147:1–12. https://doi.org/10.1016/j.pep.2018.02.005.

[29] Panhölzl J, Lindenberger J, Menczik P, Aleschko M, Hahn I, Schwartz-Zimmermann H, Schatzmayr G, Thamhesl M, Moll W. Effect of gene copy number on production yield of recombinant ergopeptine hydrolase ErgA in *Pichia pastoris*. New Biotechnol 2014;31:S188. https://doi.org/10.1016/j.nbt.2014.05.928.

[30] Ziemons S, Koutsantas K, Becker K, Dahlmann T, Kück U. Penicillin production in industrial strain *Penicillium chrysogenum* P2niaD18 is not dependent on the copy number of biosynthesis genes. BMC Biotechnol 2017;17:16. https://doi.org/10.1186/s12896-017-0335-8.

[31] Orlean P, Funai D. Priming and elongation of chitin chains: Implications for chitin synthase mechanism. Cell Surf 2019;5:100017. https://doi.org/10.1016/j.tcsw.2018.100017.

[32] Samain E, Drouillard S, Heyraud A, Driguez H, Geremia RA. Gram-scale synthesis of recombinant chitooligosaccharides in *Escherichia coli*. Carbohydr Res 1997;302:35–42. https://doi.org/10.1016/S0008-6215(97)00107-9.

[33] Coussement P, Bauwens D, Peters G, Maertens J, De-Mey M. Mapping and refactoring pathway control through metabolic and protein engineering: the hexosamine biosynthesis pathway. Biotechnol Adv 2020;40:107512. https://doi.org/10.1016/j.biotechadv.2020.107512.

[34] Rodríguez-Díaz J, Rubio-del-Campo A, Yebra MJ. Metabolic engineering of *Lactobacillus casei* for production of UDP-N-acetylglucosamine. Biotechnol Bioeng 2012;109:1704–12. https://doi.org/10.1002/bit.24475.

[35] Woo JE, Seong HJ, Lee SY, Jang Y. Metabolic engineering of *Escherichia coli* for the production of hyaluronic acid from glucose and galactose,". Front Bioeng Biotechnol 2019;7:351. https://doi.org/10.3389/fbioe.2019.00351.

[36] Nishimoto M, Kitaoka M. Identification of N-acetylhexosamine 1-kinase in the complete lacto-N-biose I/galacto-N-biose metabolic pathway in *Bifidobacterium longum*. Appl Environ Microbiol 2007;73:6444–9. https://doi.org/10.1128/aem.01425-07.

[37] Zhang W, Liu Y, Ma J, Yan Q, Jiang Z, Yang S. Biochemical characterization of a bifunctional chitinase/lysozyme from Streptomyces sampsonii suitable for N-acetyl chitobiose production. Biotechnol Lett 2020;42:1489–99. https://doi.org/10.1007/s10529-020-02834-z.

[38] Moon C, Seo D, Song Y, Hong S, Choi S, Jung W. Antifungal activity and patterns of N-acetyl-chitooligosaccharide degradation via chitinase produced from Serratia marcescens PRNK-1. Microb Pathog 2017;113:218–24. https://doi.org/10.1016/j.micpath.2017.10.039.