Zhao *et al. Microbial Cell Factories* (2025) 24:24
https://doi.org/10.1186/s12934-024-02624-6

Microbial Cell Factories

RESEARCH Open Access

Inducible engineering precursor metabolic flux for synthesizing hyaluronic acid of customized molecular weight in *Streptococcus zooepidemicus*

Rui Zhao[1], Jun Li[1], Yingtian Li[1], Xujuan Pei[1], Jingyi Di[1], Zhoujie Xie[1], Hao Liu[1*] and Weixia Gao[1*]

Abstract

Background Hyaluronic acid (HA) is extensively employed in various fields such as medicine, cosmetics, food, etc. The molecular weight (MW) of HA is crucial for its biological functions. *Streptococcus zooepidemicus*, a prominent HA industrial producer, naturally synthetizes HA with high MW. Currently, few effective approaches exist for the direct and precise regulation of HA MW through a one-step fermentation process, and *S. zooepidemicus* lacks metabolic regulatory elements with varying intensities. The ratio of HA's precursors, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcA), is critical for the extension and release of HA. An imbalance in the precursor proportions for HA synthesis leads to a significant decrease in HA MW, indicating that controlling the precursor ratio may serve as a potential method for regulating HA MW.

Results In this study, the type and concentration of carbon sources were manipulated to disrupt the balance of precursor supply. Based on the results, it was speculated that the transcription level of *hasE*, which may connect the two HA synthesis precursors, is positively correlated with HA MW. Consequently, an endogenous expression component library for *S. zooepidemicus* was constructed, comprising 32 constitutive and 4 inducible expression elements. The expression of *hasE* was subsequently regulated in strain SE0 (S12 Δ*hasE*) using two constitutive promoters of differing strengths. The recombinant strain SE1, in which *hasE* was controlled by the stronger promoter PR31, produced HA with a MW of 1.96 MDa. In contrast, SE2, utilizing the weaker promoter PR22, synthesized shorter HA with a MW of 1.63 MDa, thereby verifying the hypothesis. Finally, to precisely regulate HA MW according to specific demands, an efficient sucrose-induced expression system was screened and employed to control the transcription level of *hasE*, obtaining recombinant strain SE3. When induced with sucrose concentrations of 3, 5–10 g/L, the HA MW of SE3 reached 0.78 to 1.77 MDa, respectively.

Conclusions Studies on regulating the balance of the HA precursor substances indicate that an oversupply of either UDP-GlcNAc or UDP-GlcUA can reduce HA MW. The *hasE* gene serves as a crucial regulator for maintaining

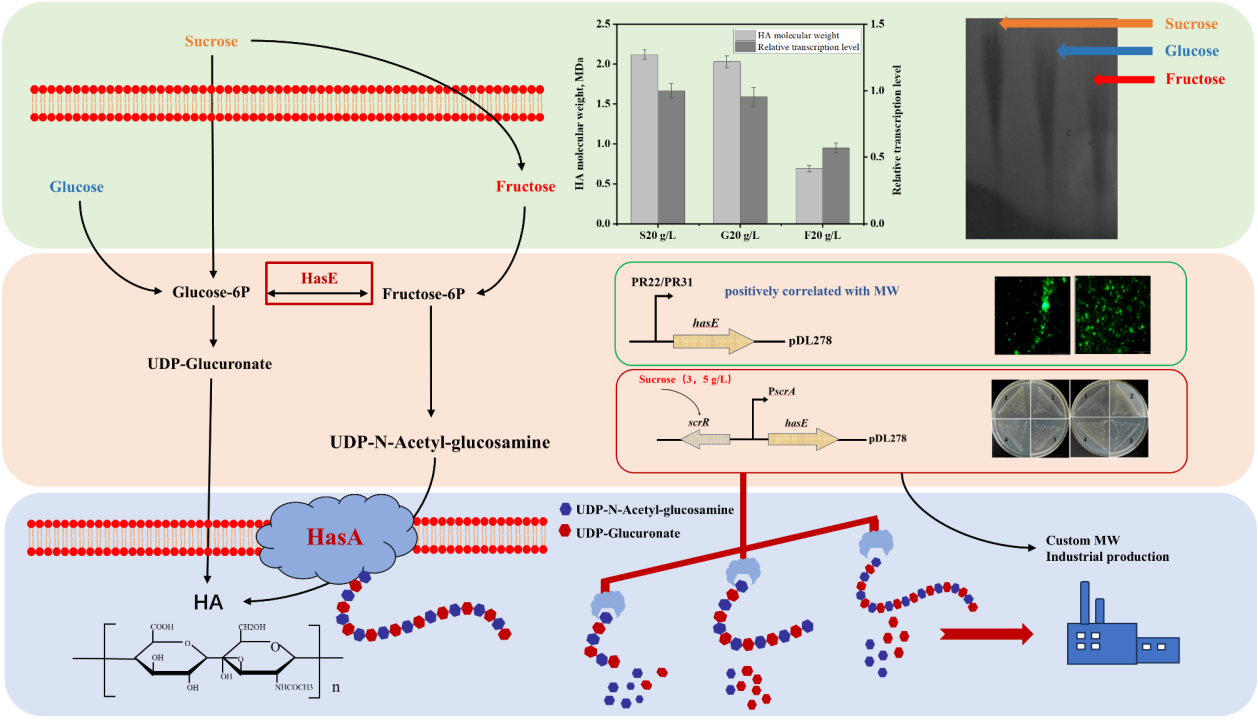
*Correspondence:
Hao Liu
liuhao@tust.edu.cn
Weixia Gao
gaoweixia@tust.edu.cn

Full list of author information is available at the end of the article

this balance. Precise regulation of *hasE* transcription was achieved through an efficient inducible expression system, enabling the customized production of HA with specific MW. The HA MW of strain SE3 can be accurately manipulated by adjusting sucrose concentration, establishing a novel strategy for customized HA fermentation.

Keywords *Streptococcus zooepidemicus*, Hyaluronic acid, Precursor supply balance, Sucrose-induced expression system, Molecular weight regulation, *hasE* gene

Graphical Abstract



Background

HA is a linear polymer consisting of a repeating disaccharide of N acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA), linked by alternating β-1,3 and β-1,4 glycosidic bonds [1]. This biopolymer is abundantly present in various human and animal tissues, as well as in microbial capsules [2]. The biological activity of HA is closely related to its molecular weight (MW) [3]. High molecular weight hyaluronic acid (HMW-HA) finds wide applications in skin moisturization [4], maintenance of viscoelasticity in ophthalmic surgery [5], intra-articular injections, cosmetics [6], skin burn healing [7], and postsurgical anti-adhesion [8]. Low molecular weight hyaluronic acid (LMW-HA) exhibits anti-inflammatory, angiogenic, and immunomodulatory effects, with HA oligosaccharides being employed in cancer treatment [9–11]. Due to its extensive biological applications, numerous studies have been conducted on the microbial synthesis of HA.

Streptococcus zooepidemicus is capable of producing HMW-HA at high yields [12–14]. Its short fermentation cycle, reduced by-product formation, high purity, and ease of separation and purification have made *S. zooepidemicus* a popular choice for HA manufacturing [15, 16]. Furthermore, the HA synthesis pathway in *S. zooepidemicus* has been well characterized, and effective gene editing tools are available to facilitate metabolic modifications [17]. However, several limitations remain in the production of HA through *S. zooepidemicus* fermentation. The organism naturally produces HA with a MW of 2.12 MDa, which does not fulfill the industrial demand for LMW-HA. Various fermentation conditions such as pH [18], temperature [19], dissolved oxygen [20], additional materials [21], and carbon/nitrogen ratio [22, 23] have been investigated to modulate the MW of HA.

Nevertheless, the MW range of HA obtained through these methods is narrow, making fine regulation challenging. Thus, an alternative approach is required for the regulation of HA MW. Generally, there has been limited research on the metabolic engineering of *S. zooepidemicus* for regulating HA MW, and there is a lack of precisely regulated metabolic modification elements. Therefore, it is essential to establish a method for the direct production of HA with specific MWs through

one-step fermentation, as well as to develop a production strain capable of fine-tuning HA MW.

Reports indicated that HA is synthesized and transported outside the cell simultaneously [24]. The competition between UDP-GlcUA and UDP-GlcNAc influences the polymerization rate by streptococcal HA synthase [25, 26]. A nearly 1:1 ratio of UDP-GlcUA to UDP-GlcNAc is advantageous for extending long HA chains and forming higher MW HA [12]. It is speculated that a disruption in the balance of UDP-GlcUA and UDP-GlcNAc may lead to premature release of HA outside the cell, resulting in the synthesis of LMW-HA.

D. Rigo et al. reported that in *Streptococcus bacterium*, the two precursors for HA synthesis are derived from glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P), respectively [3]. Will the substrate balance be modified along with the change of carbon source, such as glucose, fructose or sucrose, thereby affecting the MW of HA? In order to obtain desirable MW of HA, how to regulate the balance of G-6-P and F-6-P? In this study, various carbon sources were utilized for HA fermentation, revealing a positive correlation between *hasE* transcription levels and HA MW. Furthermore, an endogenous expression component library for *S. zooepidemicus* was constructed, and an inducible expression system was screened and employed to control *hasE*'s transcription level, aiming to regulate HA MW.

Methods

Strains and culture conditions

All strains used in this study are listed in Table 1. *S. zooepidemicus* ATCC 39,920(S12)and its derivatives were cultured at 37°C in Todd-Hewitt yeast (THY) medium [17]. *Escherichia coli* JM109 was employed for plasmid construction and was cultured in LB medium at 37 °C. Media were supplemented with spectinomycin (50 μg/mL for *E. coli*, and 100 μg/mL for *S. zooepidemicus*) when required. *Bacillus subtilis* 168 was cultured in LB medium at 37 °C, while *Lactobacillus lactis*CV56 was cultured in M17 medium at 37 °C. For HA production, *S. zooepidemicus* was cultured in HA fermentation (FSB) medium following our previously reported protocols

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Sources
E. coli JM109	host for cloning	Gibco BRL
S. zooepidemicus ATCC39920	Wild-type strain	ATCC 39,920
SA	*hasA* mutant	[34]
SE0	*hasE* mutant	This work
SA1	*hasA* mutant harboring pLH5001	This work
SA2	*hasA* mutant harboring pLH5002	This work
SA3	*hasA* mutant harboring pLH5003	This work
SA4	*hasA* mutant harboring pLH5004	This work
SE1	*hasE* mutant harboring pLH5005	This work
SE2	*hasE* mutant harboring pLH5006	This work
SE3	*hasE* mutant harboring pLH5007	This work
SPR0-32	*S12* harboring pLH5009-pLH5041	This work
Plasmid		
pDL278	Spcr, inducible expression vector	This work
pLH5001	Spc, inducible expression vector, carrying the P*scrA* promoter and *scrR* gene, derivative containing a functional *S. zooepidemicus hasA* gene	This work
pLH5002	Spcr, inducible expression vector, carrying the P*lacA* promoter and *lacR* gene, derivative containing a functional *S. zooepidemicus hasA* gene	This work
pLH5003	Spcr, inducible expression vector, carrying the P*xylA* promoter and *xylR* gene, derivative containing a functional *S. zooepidemicus hasA* gene	This work
pLH5004	Spcr, inducible expression vector, carrying the P*nisin* promote, derivative containing a functional *S. zooepidemicus hasA* gene	This work
pLH5005	Spcr, inducible expression vector, carrying the P*Sesec_00510* promote, derivative containing a functional S. zooepidemicus *hasE* gene	This work
pLH5006	Spcr, inducible expression vector, carrying the P*pfK* promote, derivative containing a functional *S. zooepidemicus hasE* gene	This work
pLH5007	Spcr, inducible expression vector, carrying the P*scrA* promoter and *scrR* gene, derivative containing a functional *S. zooepidemicus hasE* gene	This work
pLH5008	pDL278 carrying *rbs::gfpmut2* gene	This work
pLH5009-5041	pLH5008 carrying *PR0-PR32* expression element	This work

[27]. Unless otherwise specified, the carbon source for fermentation was supplied as 20 g/L sucrose.

DNA fragment techniques and cloning procedures

DNA involved in *hasE*, PR1-PR32, sucrose-inducible expression system and lactose-inducible expression system of *S. zooepidemicus*, the xylose-induced expression system of *B.subtilis* 168, nisin-induced expression system of *L. lactis* CV56, the *gfp* gene of pLH1096 (Preserved in our laboratory) were amplified from the respective genomes using specific primers (Table. S1) and Platinum Taq DNA polymerase (Takara, Dalian, China). The PCR products were purified using the QIAquick PCR purification kit (Takara, Dalian, China) and subsequently cloned into the pDL278 vector using standard T4 ligase (Vazyme, Nanjing, China). Constructs were confirmed by Sanger DNA sequencing performed by Kim Vickers and then transformed into *S. zooepidemicus* using the electroporation method as previously described [17].

Construction of an endogenous expression component library and 4 inducible expression systems for *S. zooepidemicus*

To construct an endogenous expression component library for *S. zooepidemicus*, a wild-type *S. zooepidemicus* (S12) mRNA sample was submitted to Majorbio (Shanghai, China) for sequencing analysis. The transcription levels of various genes were evaluated by calculating FPKM (fragments per kilobase of transcript per million mapped reads) values. By integrating promoter prediction scores and ribosome binding site (RBS) analysis, 32 combinations of promoters and RBSs (PR) were selected. These constitutive expression elements were then fused with the *gfp* gene for expression, and samples were fermented in FSB medium for 8 h. Fluorescence levels were detected using an upright fluorescence microscope (Olympus, Japan), with an excitation wavelength of 485 nm and an emission wavelength of 528 nm, to comprehensively assess the expression intensity of *gfp*.

The inducible expression systems were transformed into *hasA* knockout strain [34], obtaining SA1 (sucrose-inducible expression system), SA2 (lactose-inducible expression system), SA3 (xylose-inducible expression system), and SA4 (nisin-inducible expression system). The mutant strains SA1, SA2, SA3 and SA4 were streaked on THY solid plates supplemented with different concentrations of sucrose, lactose, xylose, and nisin, respectively, and incubated statically at 37 °C for 24 h.

qRT-PCR analysis

The total RNA of the target strains cultured in THY medium for 8 h was extracted using Bacterial RNA Kit (Omega Bio-tech, Guangzhou, China). The cDNA was obtained using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Transcriptional levels of the target genes were normalized against the levels of *ldh*, which encodes lactate dehydrogenase.

Growth and fermentation

S. zooepidemicus S12 and its mutant strains were fermented under shaking flask conditions [28] and the production performance of SE3 was evaluated in a 5 L fermenter. The strain was precultured in THY medium incubated at 37 °C with shaking at 200 r/min for 12 h to prepare seed cultures. For flask cultures, 500 mL flasks containing 100 mL THY medium were inoculated with 4 mL seed culture and incubated at 37 °C with shaking at 200 r/min. Fed-batch fermentation was performed in a 5 L fermenter (Parallel, Shanghai, China) containing 3 L of FSB medium, with an initial concentration of 20 g/L fructose. A 10% (v/v) inoculum from the 12-hour seed culture was used. Fermentation was performed at 37 °C with an aeration rate of 1.5 vvm and the pH was maintained at 6.8 by addition of 4 M NaOH. The agitation speed was set at 200 r/min. Sucrose was exponentially fed at rates of 3–5 g/L at 8 h. Biomass was analyzed using a spectrophotometer at 600 nm.

Analytical measurements

HA samples were purified from the broth by mixing 4 mL culture with 4 mL 0.1% w/v SDS and incubating at room temperature for 10 min. The samples were filtered through a centrifuge (Beckman Coulter, Avanti J-26 XPI) at 8000r/min for 15 min at 4 °C, the mixed with 3 volumes of ethanol and laid quietly for 12 h at 4 °C. Then mixture was centrifuged (8000 r/min for 20 min at 4 °C), and the pellet was allowed to dry overnight. The dried HA pellet was resuspended in 0.1 M $NaNO_3$ with gentle rocking overnight at 4 °C. Prior to analysis, samples were filtered through a 0.22 μm filter (Millex-GS MSE).

Purified HA samples were analyzed for their concentration and MW, using the turbidimetric quantification assay [29] and agarose gel electrophoresis [30], respectively. For gel electrophoresis, samples were separated with a Sub-Cell GT (Bio-Rad) apparatus and 0.5% agarose gels. Samples containing 0.5 g/L HA were electrophoresed at 35 mA for 1 h, followed by a switch to a constant current of 80 mA for 3 h at room temperature. The gels were stained with toluidine blue as previously described [31], and the samples were then filtered through a syringe filter (Millex-GS MSE, 0.45 μm) to remove cells. HA MW was measured by GPC, as described elsewhere [31].

Assay of biofilm

A single colony of *S. zooepidemicus* was inoculated into 4 mL of THY liquid medium and cultured overnight at 37 °C with agitation at 200 r/min. For crystal violet test, 200 μL of the bacterial solution was added to a 96-well

plate and incubated at 37 °C for 24 h. The cells were rinsed three times with PBS. Subsequently, 200 μL of methanol was added to each well to fix the cells at room temperature for 30 min and then dried in an oven. Next, 200 μL of 1% crystal violet solution was added to each well, and the cells were stained for 30 min. The 96-well plate was rinsed three times with PBS and air-dried. Finally, OD_{595} was measured after adding 200 μL of 33% glacial acetic acid solution to each well to dissolve the adherent bacteria.

For Congo red test, the tested bacterial solution was dripped to corresponding Congo red plates and marked. Three repetitions were performed. The plates were then incubated at a constant temperature of 37 °C for 24 h, after which the colonies were photographed to observe their color.

Results and discussion

Effects of carbon source supply on HA MW

Studies have indicated that feeding sucrose in the fermentation medium can increase the MW of HA by 800 kDa compared to using glucose as a carbon source [32]. The exact mechanism underlying this phenomenon is unclear. To investigate the effects of varying carbon sources on HA MW, a fermentation medium supplemented with 20 g/L of sucrose, glucose, or fructose, respectively, was used for HA production. The MW of HA reached up to 2.12 MDa, 2.06 MDa, and 0.69 MDa when fermentation with sucrose, glucose, and fructose, respectively (Fig. 1A and B). Moreover, the HA MW only showed a slight decrease with the reduction of glucose concentration, but this difference was not significant. (Figure 1C and D). However, it was observed that the lower MW obtained with *S. zooepidemicus* could be further reduced by decreasing the concentration of fructose. When the fructose concentration was lowered to 5 g/L, the HA MW reached its lowest level of 0.42 MDa (Fig. 1E and F).

As we know, the synthesis of HA precursor involves two metabolic pathways: G-6-P and F-6-P. In the former pathway, the G-6-P is converted to UDP-GlcUA via the enzymes PgmA, HasC, and HasB. In the latter pathway, GlmS, GlmM, and HasD convert F-6-P to UDP-GlcNAc. When sucrose or glucose is utilized as the carbon source, HasE acts as a bridge between F-6-P and G-6-P, with G-6-P becoming the primary source of F-6-P. Furthermore, fructose can be converted to F-6-P by ScrK, providing additional carbon sources for UDP-GlcNAc synthesis. Subsequently, HasA converts UDP-GlcUA and UDP-GlcNAc to HA. Fructose and F-6-P activates the EMP metabolic pathway via *fruA* and *pfk*, respectively, while UDP-GlcNAc serves as a precursor for cell wall fabrication. Consequently, the biosynthesis of UDP-GlcNAc competes for resources with the EMP pathway and the cell wall synthesis pathway [33]. When sucrose serves as the carbon source, it can simultaneously support the synthesis of G-6-P and fructose, thus enabling the precursors to maintain an optimal ratio, which favors the synthesis of HMW-HA. Conversely, the employment of glucose or fructose as carbon sources disrupts this precursor balance, resulting in the production of LMW-HA.

Previous reports indicated that UDP-GlcNAc plays a decisive role in the MW of HA [24]. It is speculated that the expression level of *hasE* and *scrK* might significantly impact the precursor balance, subsequently affecting the MW of the HA polymer chain. To confirm this effect, we compared the transcript levels of *hasE* and *scrK* in different carbon sources. Compared to 5 g/L fructose, 20 g/L resulted in a dramatic increase in *hasE* transcript levels of 15.46-fold (Fig. 1D), while no significant difference was noted for *scrK* (Fig. S1). This suggested that HA MW is correlated with *hasE* transcript levels, indicating that all F-6-P is converted to G-6-P primarily via *hasE*. It can be considered that the HA MW correlated with *hasE* transcript level. Therefore, it can be considered that all F-6-P is converted to G-6-P solely by *hasE*. Strains fed with fructose exhibited suboptimal levels of UDP-GlcUA compared to UDP-GlcNAc, highlighting a strong correlation between MW and UDP-GlcUA concentration. In summary, an excess of either precursor can disrupt substrate balance, leading to the production of LMW-HA.

As fructose concentration decreases, the cellular growth rate slows, making it an ineffective strategy to restrict *hasE* gene transcription by limiting fructose concentration. Consequently, relying solely on reduced fructose levels to adjust HA MW is not a practical approach, necessitating the exploration of alternative strategies.

Effect of *hasE* knockout on HA MW

Previous studies have demonstrated that, in addition to glycolysis pathway, phosphate glucose isomerase, encoded by *hasE*, is a key enzyme in the biosynthesis of HA in *S. zooepidemicus* and was knocked out in our previous studies [34]. It was observed that the colony surface of SE0 (S12 Δ*hasE*) exhibits a dry and loose appearance, accompanied by an absence of discernible capsule formation [34]. In this study, it was found that the strain SE0 (S12 Δ*hasE*) significantly contributed to a decrease in HA MW compared to S12, with HA MW dropping to 0.33 MDa (Fig. 2). We speculated that the knockout of *hasE* might completely change the precursor supply balance of HA, potentially leading to premature release of the HA polymer chain. This suggests that modulation of the ratio of G-6-P and F-6-P through *hasE* transcription levels would be a feasible method to adjust precursor balance and regulate HA MW. During the fermentation process, we observed slow growth in SE0 (S12Δ*hasE*) when sucrose was used as the carbon source (Fig. S2). However,

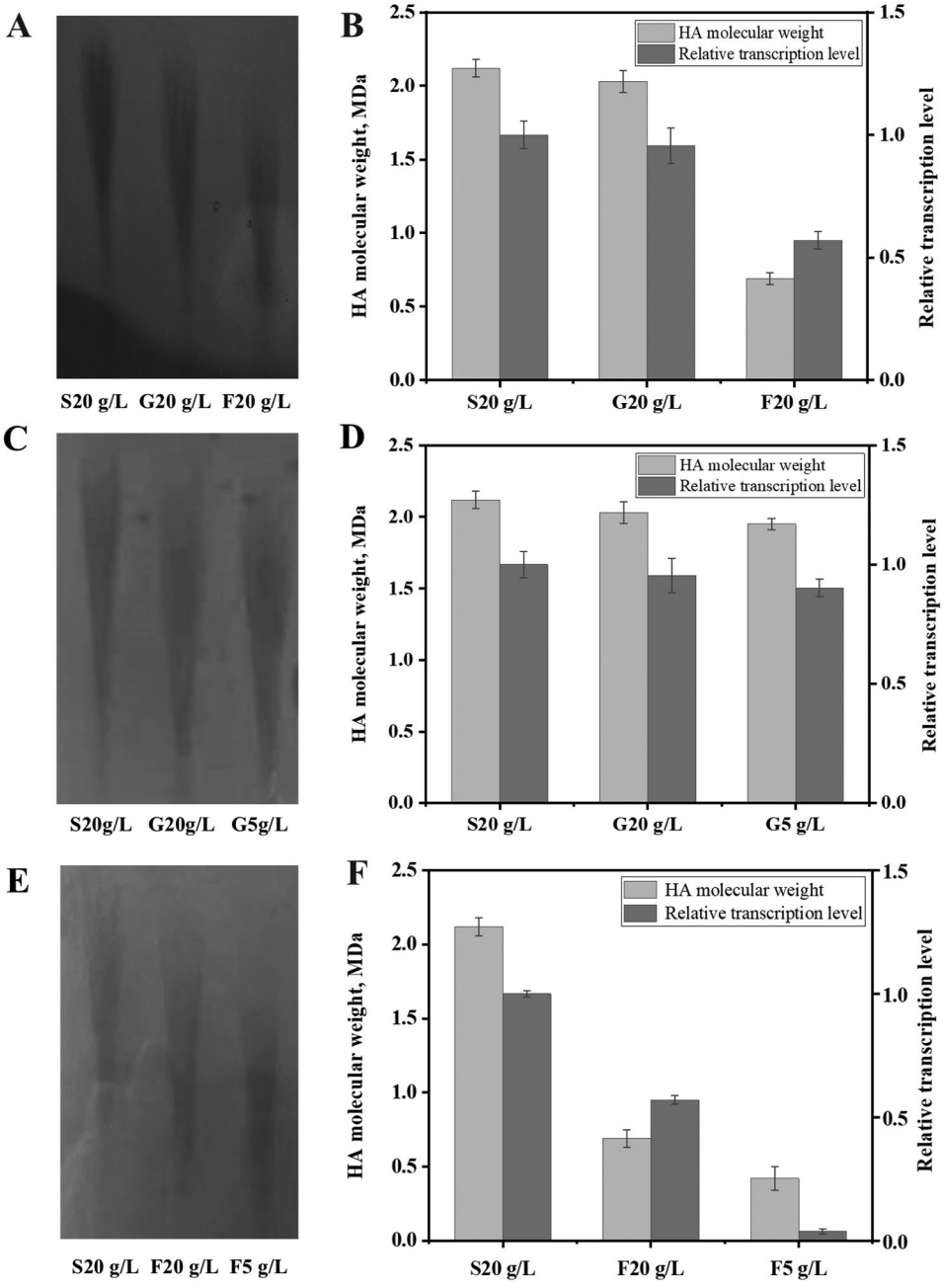


Fig. 1 The gel electrophoresis of HA (**A**), HA MW and *hasE* transcription levels (**B**) after 24 h of culture feeding 20 g/L sucrose, glucose, and fructose as carbon sources, respectively. The gel electrophoresis of HA (**C**), HA MW and *hasE* transcription levels (**D**) after 24 h of culture feeding 20 g/L sucrose, 5 g/L and 20 g/L glucose as carbon sources, respectively. The gel electrophoresis of HA (**E**), HA MW and *hasE* transcription levels (**F**) after 24 h of culture feeding 20 g/L sucrose, 5 g/L and 20 g/L fructose as carbon sources, respectively

by feeding fructose, the mutant strain SE0 exhibited growth recovery(Fig. S2)and a small amount of capsule were observed on the colony. Hence, fructose serves as a supplementary carbon source for strain growth, effectively enhancing the availability of essential precursors for UDP-GlcNAc biosynthesis.

Regulation of *hasE* transcript levels using different strength constitutive promoters

As previously mentioned, solely reducing the fructose concentration to manipulate the HA MW is not feasible. Regulating the expression level of *hasE* through synthetic biology approaches may present a more effective solution. In contrast to widely utilized model microorganisms, such as *E. coli* [35] and *B. subtilis* [36], *S.*

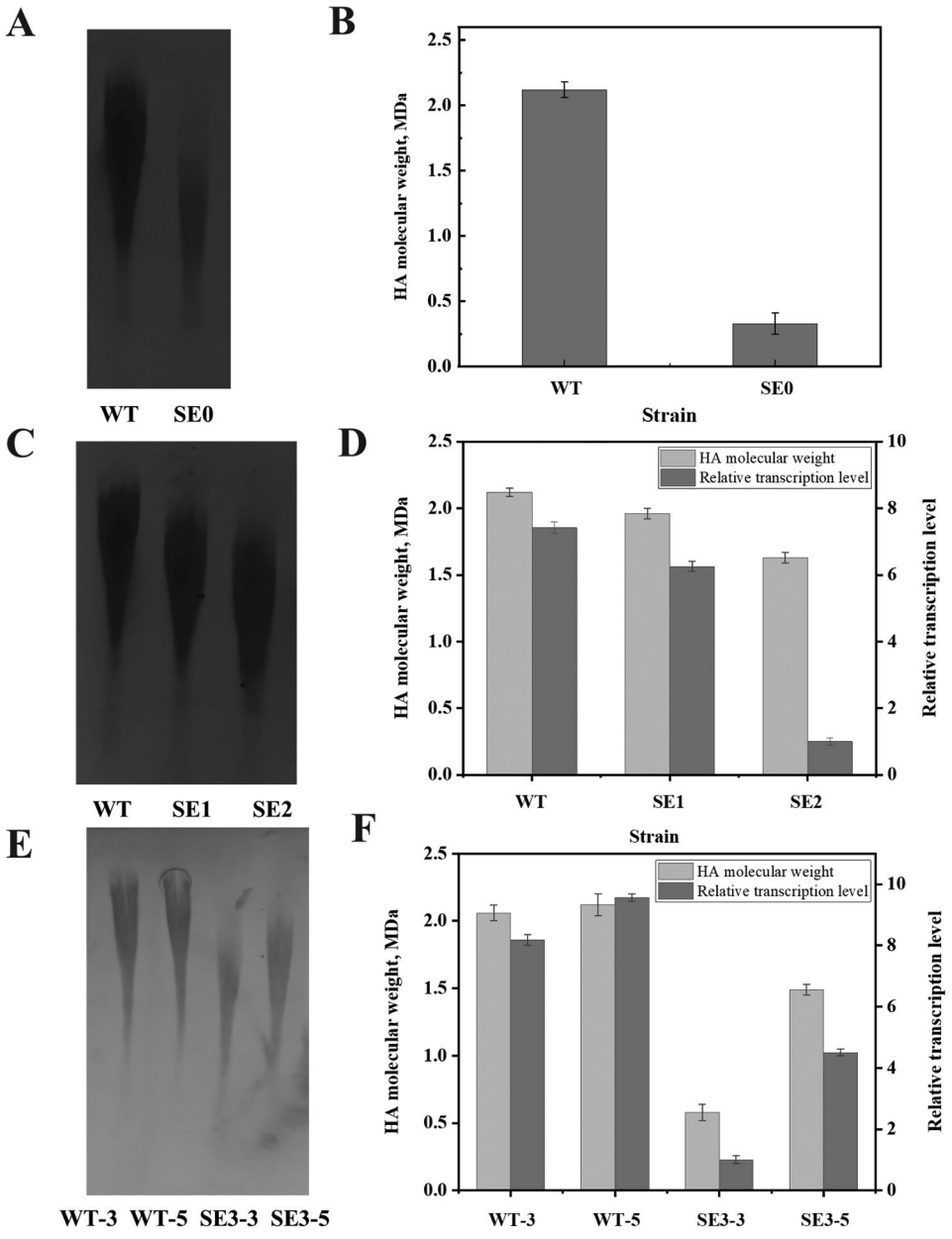


Fig. 2 The changes of the HA MW after knockout of the *hasE* gene. The gel electrophoresis of HA (**A**) and HA MW (**B**) of the S12 and SE0 (S12 Δ*hasE*) after 24 h of culture using 20 g/L sucrose as the sole carbon source. The gel electrophoresis of HA (**C**), HA MW and *hasE* transcription levels (**D**) of the WT, SE1 and SE2 after 24 h of culture using 20 g/L sucrose as carbon source. The gel electrophoresis of HA (**E**), HA MW and *hasE* transcription levels (**F**) of the WT and SE3 after 24 h of culture feeding 20 g/L fructose as carbon source, adding 3 g/L and 5 g/L of sucrose as inducers, respectively

zooepidemicus notably lacks potent expression elements, posing significant challenges for metabolic engineering efforts. The promoter cp25, derived from *Streptomyces*, has been shown to function in *S. zooepidemicus*; however, its expression strength is significantly lower than desired levels [37], limiting its practical utility in metabolic engineering applications. To address this, 32 feasible constitutive expression elements have been screened (Fig. 3) to regulate *hasE* expression. All fluorescent images and sequences can be seen in Fig. S3 and Sequence. S1. The constructed *hasE* expression plasmid, under the control of the constitutive promoter PR22 (medium strength) and PR31 (high strength), was transformed into strain SE0 (S12), obtaining SE1 and SE2. As expected, compared to SE2, the transcription level of *hasE* in SE1 increased by 6.26-fold, leading to an increase in the HA MW from 1.63 MDa to 1.96 MDa. (Figure 2C and D). These results suggest that the MW of HA increases with the enhancement of promoter strength, indicating that a promoter replacement strategy could effectively regulate HA MW.

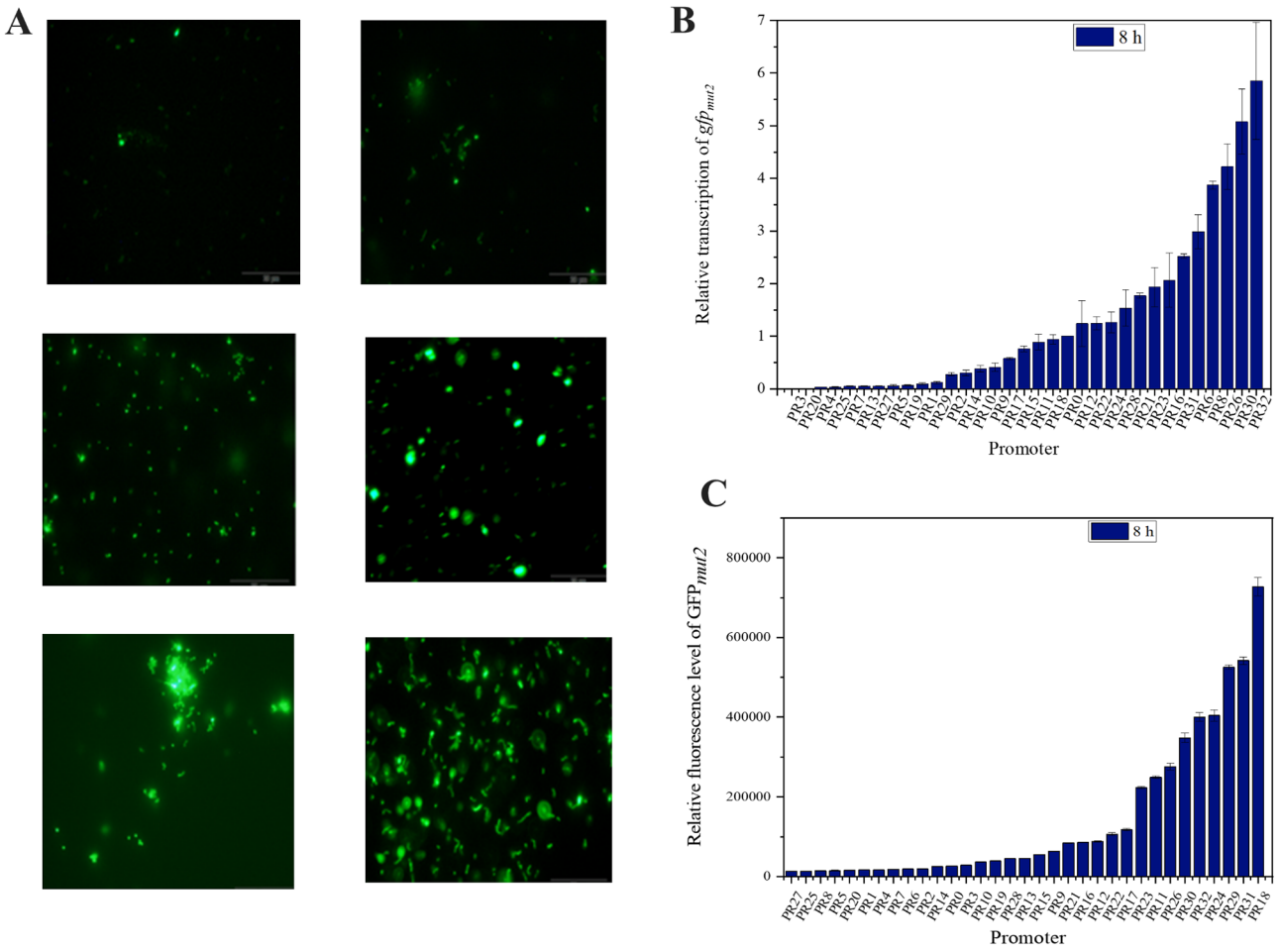


Fig. 3 After 8 h of fermentation using FSB medium, fluorescent images (**A**) of some representative gradient-intensity constitutive expression elements. The relative transcription levels (**B**) and fluorescent expression levels (**C**) of 32 expression elements.

Screening of inducible expression systems

To accomplish the fine-tuned manipulation of HA MW, a controllable and potent inducible expression system is required. However, given the lack of native inducible expression elements in *S. zooepidemicus*, we screened four homologous or heterologous inducible expression components. Analysis of the *S. zooepidemicus* genome revealed that the sucrose operon primarily comprises *scrR* (repressor of the sucrose operon), *scrB* (sucrose-6-phosphate hydrolase), and *scrA* (component of the IIABC of the sucrose PTS) [38]. Based on this structure, it is speculated that the sucrose promoter might be activated by sucrose in a way similar to lactose operon promoters, with *scrR* positioned opposite to *scrA* (Fig. S4A). To validate the activity of the P_{scrA}-*scrR* construct, we created a gene fusion between the promoter region of *scrA* and *scrR* to test whether the *scrA* promoter can initiate the transcription of *hasA* (Hyaluronic Acid Synthase coding gene) and under the regulation of *scrR*. The open reading frame (ORF) for such a gene fusion is illustrated in Fig. S4A. Besides, three previously reported inducible expression systems, lactose, xylose, and nisin [39], were characterized using the same approach. It was observed that the mutant strain SA1 formed a plump capsule with moist, smooth, round, and raised colonies. In contrast, SA2 generated a thin capsule with relatively smooth and moist colonies that were slightly raised, while SA3 and SA4 did not form any capsule, and their colonies appeared dry and loose (Fig. S4B).

Consistently with the colony morpholog, SA1 induced by sucrose concentrations of 0, 1, 5, and 10 g/L produced HA at yields of 0.196, 0.578, and 0.712 g/L, respectively. Under the condition of 10 g/L sucrose induction, the relative transcription level of the *hasA* gene was 23 folds higher than that under 1 g/L. Overall, these findings indicate that the sucrose-inducible expression system can efficiently induce gene expression in *S. zooepidemicus*.

Regulation of *hasE* transcript levels using sucrose inducible expression system

The constructed *hasE* expression plasmid, under the control of sucrose-induced expression system, was

transformed into strain SE0 (S12 Δ*hasE*), resulting in strain SE3. We employed this expression system (SE3) with varying initial sucrose concentrations to regulate HA MW. When the sucrose concentration rose from 3 g/L to 5 g/L, the HA MW elevated from 0.58 MDa to 1.49 MDa, accompanied by a significant 4.5-fold increase in the expression level of *hasE* (Fig. 2E and F). Conversely, the HA MW in strain S12 exhibited a slight increase with rising sucrose concentration (Fig. 2E and F).

These results suggest a positive correlation among sucrose concentration, *hasE* transcription level, and the MW. Therefore, it is possible and desirable to produce HA with various MW by precisely controlling the expression levels of *hasE* by artificially adding different concentrations of sucrose. In summary, a sucrose-induced expression system was employed to optimize *hasE* expression and modulate the MW of HA. Sucrose can also accumulate extra G-6-P for HA synthesis, leading to suboptimal levels of UDP-GlcNAc of SE3.

Biofilm formation

To investigate precursor balance as a potential control point of capsule generation, we performed Congo red and crystal violet tests on strains S12, SE0, and SE3 (with sucrose concentrations of 0, 3 and 5 g/L). Compared to S12, the *hasE* knockout strain SE0 displayed a marked difference in rdar morphology, exhibiting a distinct dry red colony phenotype (Fig. 4A). These results indicated that *hasE* gene knockout significantly reduced the biofilm formation ability of *S. zooepidemicus*. Furthermore, the fermentation results for SE3 revealed that at lower initial sucrose concentrations, there was an inhibitory effect on biofilm formation. However, the rdar morphology recovered with an increasing substrate concentration from 0 to 5 g/L. A similar trend was observed in the crystal violet assay (Fig. 4B and C). In biofilm formation processes, lower transcription levels of *hasE* result in a decreased proportion of HMW-HA in the capsule of *S. zooepidemicus*, ultimately leading to the development of a thin

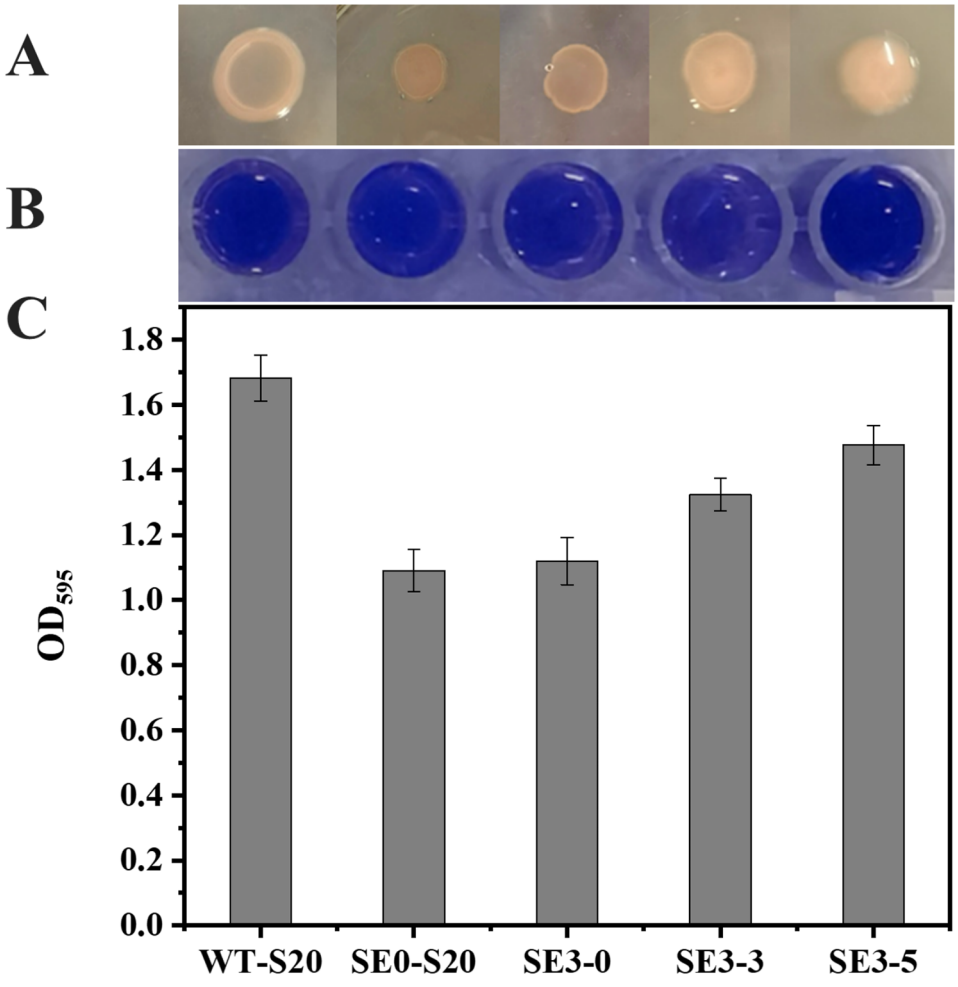


Fig. 4 Verification of the effect of substrate balance on the biofilm of strains through congo red staining (**A**) and crystal violet test (**B**, **C**). The positive control was the S12 supplemented with 20 g/L sucrose (WT-S20), the negative control was the *hasE* knockout strain supplemented with 20 g/L sucrose (SE0-S20), and the experimental groups were SE3 strains induced with 0 g/L (SE3-0), 3 g/L (SE3-3), and 5 g/L (SE3-5) sucrose, respectively

and feeble capsule. It can be seen that the viscosity of *S. zooepidemicus* decreased significantly, which facilitated increased dissolved oxygen content during fermentation, thereby enhancing strain density and HA yield.

5 L fermentation of SE3

To evaluate the production performance, the engineered *S. zooepidemicus* strain SE3 was assessed in a 5 L fermenter. As illustrated in Fig. 5, the HA MW were 0.78MDa, 1.66MDa, and 1.77MDa at sucrose concentrations of 3 g/L, 5 g/L, and 10 g/L, respectively. As shown in Fig. 5A and B, the admixture of fructose and sucrose in S12 lead to the generation of HMW-HA, Therefore, LMW-HA could not be obtained in S12 through the strategy of mixing carbon sources. When only 20 g/L of fructose is fed to S12, although the MW of HA was reduced to 0.68 MDa, the obtained HA generation reached only 1.3 g/L. However, when a mixed carbon source was used, the engineered strain SE3 produced a maximum of 4.38 g/L of HA (Fig. 5C). These data suggested that, despite fructose providing sufficient carbon source for strain growth, the supply capability was insufficient to satisfy the requirements for HA synthesis. In contrast, the supplemental addition of sucrose in SE3 not only afforded precise control of HA MW but also provided the requisite carbon source for HA biosynthesis. In conclusion, SE3 fed with sucrose exhibited a superior production capacity compared to S12 supplemented with fructose.

Currently, numerous studies have reported on the synthesis of HA using microorganisms. Among them, the yield of HA in *Lactococcus lactis* is 1.8 g/L, with a molecular weight of 2.8 MDa [40]. In *B. subtilis*, the HA yield is approximately 1 g/L, with the molecular weight fluctuating within the range of 1.1–1.2 MDa [41]. However, the current industrial production of HA is carried out by *Streptococcus* species, mainly *S. zooepidemicus*, due to their high production of HA [3]. The HA production by wild-type *S. zooepidemicus* often falls within the range of 4–5 g/L [42, 43], close to the 4.38 g/L yield obtained in this study. Relevant reports have indicated that optimizing culturing conditions can significantly increase HA production. Pan et al. successfully achieved a HA yield of 7 g/L through a combination of optimized fermentation conditions and fermentation tank process control, demonstrating that *S. zooepidemicus* still has great potential in HA production [44]. However, when the HA content in *S. zooepidemicus* exceeds 4 g/L, the viscosity of the fermentation broth increases sharply, leading to a drastic reduction in dissolved oxygen (DO), which complicates biomass accumulation and HA production [20]. We successfully reduced the viscosity of the fermentation broth by disrupting the precursor balance for HA synthesis (Fig. 4). By further optimizing the fermentation conditions and incorporating precursor balance strategies, it is promising to achieve a higher HA yield. Compared to yield, the regulation of HA MW deserves more attention. Despite the various approaches that researchers have currently adopted to regulate HA MW, the effects are not pronounced [18–23].For example, Jagannath et al. maintained dissolved DO level at 30% saturation and supplyed a constant air flow of 1 vvm, after which the final HA titer and MW under anaerobic conditions were inferior to the control by about 13% and 17%, respectively [45]. Kumari et al. achieved the regulation of HA MW by mutating the Cys residue of hyaluronic acid synthase, resulting in an approximately 50% decrease in MW compared to the wild type [46]. In this study, a large number of highly effective constitutive and inducible expression elements were screened, and through these elements, the balance of precursor substances was regulated. Successfully, customized production of HA with different MW was achieved through a one-step fermentation process, overcoming the difficulty of the narrow regulation range of HA MW.

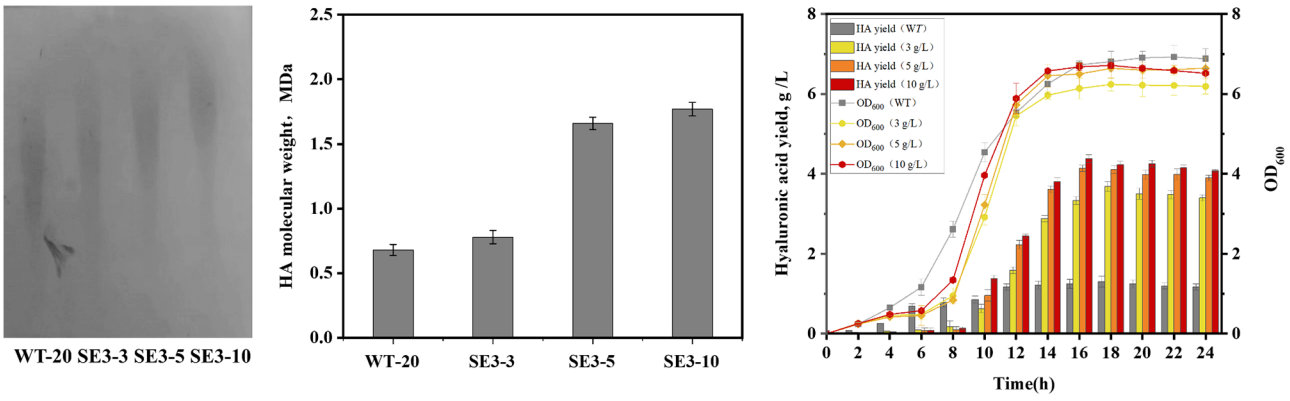


Fig. 5 The gel electrophoresis of HA (**A**), HA MW (**B**), cell growth and HA yield (**C**) after 24 h of culture in a 5L fermentor, adding 3 g/L (SE3-3), 5 g/L (SE3-5) and 10 g/L (SE3-10) sucrose as inducer, respectively. Each experimental group was added with 20 g/L fructose to provide carbon sources for the fermentation of all strains

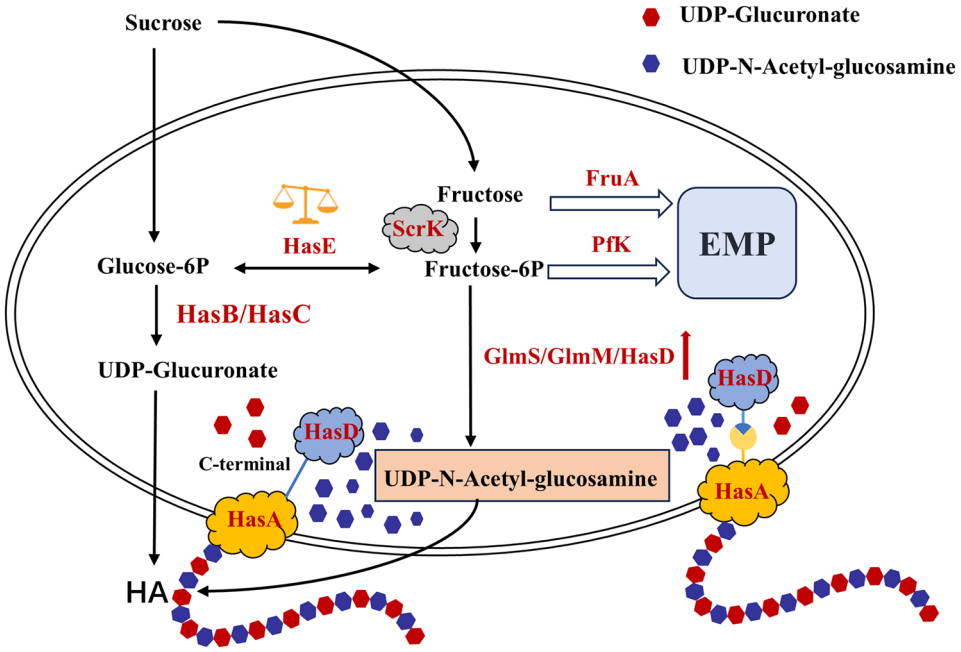


Fig. 6 The purpose of enriching UDP-GlcNAc around HasA by heterologous expression of ScrK, enhancement of UDP-GlcNAc synthesis pathway, fusion expression of HasD and HasA, and utilization of protein scaffolds

It can be observed that HA MW exhibits a regular and consistent downward trend as the concentration of sucrose increases. However, HA MW does not exhibit a strict linear correlation with the expression level of the *hasE* gene. Future research can focus on exploring more sensitive and controllable induction systems. Besides, in the industrial production of LMW-HA, the method of adding hyaluronidase to HMW-HA is commonly used currently [47]. The sucrose-inducible expression system screened in this study can be employed to regulate the expression of hyaluronidase to obtain HA with different MW. Thus, the efficient inducible expression system developed in this study offers more possibilities for customized production of HA with specific MW.

In order to further reduce the MW of HA and achieve higher yields, another more effective modification strategy urgently must be explored. Based on prior discussions, the inferior competitive position of UDP-GlcUA similarly serves as a constraint on the augmentation of HA MW. Since UDP-GlcNAc is crucial for initiating HA chain elongation, its abundance can improve the efficiency of HA synthesis, potentially leading to higher yields of LMW-HA. In conclusion, the excessive accumulation of UDP-GlcNAc is a key factor in achieving LMW-HA and a high yield of HA. Increasing UDP-GlcUA concentration is thus a feasible and effective way to control HA MW.

However, even with the overexpression of key genes in the HA synthesis pathway, such as *glmS* and *glmM* [25], it remains challenging to accumulate sufficient UDP-GlcNAc. Consequently, we need to explore additional methods to increase the dominance of UDP-GlcNAc. Among these, overexpression of *scrK* to elevate the concentration of the precursor substance F-6-P is a necessary approach. As we know, fructose can be converted to F-6-P by *ScrK*, which is considered as a supplement for HasE. However, the low expression level of *scrK* in S12 resulted in its inability to accumulate excess HA (unpublished data). Increasing *scrK* activity or transcription level would be an alternative strategy to boost F-6-P levels. Overexpressing heterologous *scrK* or screening for higher strength promoters and RBS sequences would enhance F-6-P concentration. Additionally, modulating the expression level of enzymes involved in HA synthesis can be employed to alter the substrate balance. *HasA*, located on the cell membrane, forms long chains of HA by alternately connecting UDP-GlcUA and UDP-GlcNAc. The pores created by *hasA* facilitate the transmembrane transport of HA. It has been observed that overexpressing *hasA* results in a substantial decrease in HA MW and an increase in yield, likely due to competitive binding of HA synthesis precursors [25, 48–50]. Based on this assumption, the overexpression of *hasD*, which is involved in UDP-GlcNAc synthesis, significantly influences substrate equilibrium. To further disturb the substrate balance, it could be a clever operation to fuse the *hasD* gene to the C-terminus of *hasA* or anchor *hasD* to the cell membrane through constructing protein scaffolds (Fig. 6). This strategy would bring the synthesized UDP-GlcNAc closer spatially to *hasA*, enhancing the

disruption of substrate balance at a spatial level (Fig. 6). Moreover, we have identified that the insufficient supply of amino donors limits the excessive accumulation of UDP-GlcNAc, and that promoting the utilization of glutamine can stimulate UDP-GlcNAc synthesis (data not shown). Using these strategies, UDP-GlcNAc could be turned into the preferred substrate and a high yield of LMW-HA might be achieved, which is exciting news for HA industrial production.

Conclusions

The results of this study confirmed that regulating the transcription level of *hasE* can disrupt the precursor balance during HA synthesis, thereby achieving LMW-HA production. We have constructed an expression component library for *S. zooepidemicus* and demonstrated its effectiveness in metabolic engineering. Our findings reveal that the MW of HA positively correlates with the transcription level of *hasE*, and an excess of any precursor substance can also disrupt the balance. Finally, we have established a sucrose-inducible expression system for precisely regulating the *hasE*, enabling the direct production of HA with specific MW through a simplified one-step fermentation process. In a 5 L fermenter, the engineered strain SE3 allows the generation of a maximum of 4.38 g/L HA, underscoring its efficiency and potential for industrial-scale production of HA with tailored properties.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02624-6.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Hao Liu and Weixia Gao designed the research; Rui Zhao, Jun Li, Xujuan Pei and Jingyi Di performed the research; Rui Zhao and Yingtian Li analyzed the data; and Rui Zhao and Weixia Gao wrote the paper.

Funding
This work was supported by National Key Research and Development Program of China (2021YFC2103200).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]MOE Key Laboratory of Industrial Fermentation Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, P. R. China

Received: 7 October 2024 / Accepted: 16 December 2024
Published online: 18 January 2025

References

1. Stern R, Asari AA, Sugahara K. Hyaluronan fragments: an information-rich system. Cell Biol. 2006;85(8):699–715. https://doi.org/10.1016/j.ejcb.2006.05.009.
2. Graça MFP, Miguel SP, Cabral CSD, Correia IJ. Hyaluronic acid-based wound dressings: a review. Carbohydr Polym. 2020;241:116364–417. https://doi.org/10.1016/j.carbpol.2020.116364.
3. Diane R, Leonardo M, Silva D, Fischer B. Hyaluronic acid – from production to application: a review. BRIAC. 2023;13(3):211–33. https://doi.org/10.33263/BRIAC133.211.
4. Zheng XL, Wang BT, Tang X, Mao BY, Zhang QX, Zhang TM, Zhao JX, Cui SM, Chen W. Absorption, metabolism, and functions of HA and its therapeutic prospects in combination with microorganisms: A review. Carbohydr Polym. 2023; 299:120153-71. 10.1016/ j. carbpol. 2022. 120153.
5. Johnson ME, Murphy PJ, Boulton M. Effectiveness of sodium hyaluronate eyedrops in the treatment of dry eye. Graefes Arch Clin Exp Ophthalmol. 2006;244(1):109–12. https://doi.org/10.1007/s00417-005-0028-1.
6. Altman RD, Manjoo A, Fierlinger A, Niazi F, Nicholls M. The mechanism of action for HA treatment in the osteoarthritic knee: a systematic review. BMC Musculoskelet Dis. 2015;16(1):321–31. https://doi.org/10.1186/s12891-015-0775-z.
7. Chen LH, Xue JF, Zheng ZY, Shuhaidi M, Thu HE, Hussain Z. HA, an efficient biomacromolecule for treatment of inflammatory skin and joint diseases: a review of recent developments and critical appraisal of preclinical and clinical investigations. Int J Biol Macromol. 2018;116:572–84. https://doi.org/10.1016/j.ijbiomac.2018.05.068.
8. Gunasekaran VDGVP. Role of membrane proteins in bacterial synthesis of HA and their potential in industrial production. Int J Biol Macromol. 2020;164:1916–26. https://doi.org/10.1016/j.ijbiomac.2020.08.077.
9. West DC, Kumar S. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. Exp Cell Res. 1989;183(1):179–96. https://doi.org/10.1016/0014-4827(89)90428-x.
10. Termeer C, Sleeman JP, Simon JC. Hyaluronan–magic glue for the regulation of the immune response? Trends Immunol. 2003;24(3):112–24. https://doi.org/10.1016/s1471-4906(03)00029-2.
11. Benitez A, Yates TJ, Lopez LE, Cerwinka WH, Bakkar A, Lokeshwar VB. Targeting hyaluronidase for cancer therapy: antitumor activity of sulfated HA in prostate cancer cells. Cancer Res. 2011;71(12):4085–95. https://doi.org/10.1158/0008-5472.CAN-10-4610.
12. Chen WY, Marcellin E, Steen JA, Nielsen LK. The role of hyaluronic acid precursor concentrations in molecular weight control in *Streptococcus zooepidemicus*. Mol Biotechnol. 2014;56(2):147–56. https://doi.org/10.1007/s12033-013-9690-4.
13. Tlustá M, Krahulec J, Pepeliaev S, Franke L, Cerný Z, Jílková J. Production of hyaluronic acid by mutant strains of group C *Streptococcus*. Mol Biotechnol. 2013;54(3):747–55. 10.1007/ s12033-012-9622-8.
14. Krahulec J, Tlustá M, Stuchlík S, Turňa J. Structure of the has operon promoter and the effect of mutations on the has promoter strength in Streptococcus equi subsp. zooepidemicus. Mol Biotechnol. 2011;49(2):166–75. https://doi.org/10.1007/s12033-011-9388-4.
15. Pummill PE, DeAngelis PL. Alteration of polysaccharide size distribution of a vertebrate hyaluronan synthase by mutation. J Biol Chem. 2003;278(22):19808–14. https://doi.org/10.1074/jbc.M301097200.
16. Keasling JD. Manufacturing molecules through metabolic, engineering. Science. 2010;330(6009):1355–8. https://doi.org/10.1126/science.1193990.
17. Sun XY, Yang DD, Wang YY, Geng HR, He XH, Liu H. Development of a markerless gene deletion system for *Streptococcus zooepidemicus*: functional characterization of hyaluronan synthase gene. Appl Microbiol Biotechnol. 2013;97(19):8629–36. https://doi.org/10.1007/s00253-013-5058-8.
18. Shoparwe NF, Kew WS, Mohamad M, Ameram N, Makhtar MMZ. Optimization and Kinetic Analysis on the production of Hyaluronic Acid by *Streptococcus Zooepidemicus* in a batch system. IOP Conf Ser Earth Environ Sci. 2020;596(1):12046–53. https://doi.org/10.1088/1755-1315/596/1/.

19. Cavalcanti ADD, Melo BAG, Ferreira BAM, Santana MHA. Performance of the main downstream operations on hyaluronic acid purification. Process Bioche. 2020;99:160–70. https://doi.org/10.1016/j.procbio.2020.08.020.
20. Yao ZY, Qin J, Gong JS, Ye YH, Qian JY, Li H, Xu ZH, Shi J. Versatile strategies for bioproduction of hyaluronic acid driven by synthetic biology. Carbohydr Polym. 2021;15(264):118015–39. https://doi.org/10.1016/j.carbpol.2021.118015.
21. Cheng Q, Liu C, Zhao J, Li WW, Guo FX, Qin JW, Wang YS. Unlocking the potential of hyaluronic acid: exploring its physicochemical properties, modification, and role in food applications. Trendsfood Sci Tech. 2023;14(104218):0924–2244. 10.1016/. j. tifs.2023. 104218.
22. Don MM, Noor FS. Kinetics of hyaluronic acid production by *Streptococcus zooepidemicus* considering the effect of glucose. Biochem Eng J. 2010;49(1):95–103. https://doi.org/10.1016/j.bej.2009.12.001.
23. Arslan NP, Aydogan MN. Evaluation of sheep wool protein hydrolysate and molasses as low-cost fermentation substrates for hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 35246. Waste Biomass Valori. 2021;12:925–35. https://doi.org/10.1007/s12649-020-01062-w.
24. Yang J, Cheng FY, Yu HM, Wang JT, Guo ZG, Stephanopoulos G. Key role of the Carboxyl Terminus of Hyaluronan synthase in Processive synthesis and size control of Hyaluronic Acid. Polym Biomacromolecules. 2017;18(4):1064–73. https://doi.org/10.1021/acs.biomac.6b01239.
25. Chen WY, Marcellin E, Hung J, Nielsen LK. Hyaluronan molecular weight is controlled by UDP-N-acetylglucosamine concentration in *Streptococcus zooepidemicus*. J Biol Chem. 2009;284(27):18007–14. https://doi.org/10.1074/jbc.M109.011999.
26. Badle SS, Jayaraman G, Ramachandran KB. Ratio of intracellular precursors concentration and their flux influences hyaluronic acid molecular weight in *Streptococcus zooepidemicus* and recombinant *Lactococcus lactis*. Bioresour Technol.2014; 163:222-7. 10.1016/ j. biortech. 2014. 04. 027.
27. Gao WX, Xie YY, Zuo M, Zhang GT, Liu H. Improved genetic transformation by disarmament of type II restriction-modification system in *Streptococcus zooepidemicus*. 3 Biotech. 2022;12(9):192–202. https://doi.org/10.1007/s13205-022-03227-x.
28. Sun XQ, Wang Z, Bi YL, Wang YY, Liu H. Genetic and functional characterization of the hyaluronate lyase HylB and the beta-N-acetylglucosaminidase HylZ in *Streptococcus zooepidemicus*. Curr Microbiol. 2015;70(1):35–42. https://doi.org/10.1007/s00284-014-0679-4.
29. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem. 1962;330–4. https://doi.org/10.1016/0003-2697(62)90095-7.
30. Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. Anal Biochem. 1994;219(2):278–87. https://doi.org/10.1006/abio.1994.1267.
31. Marcellin E, Gruber CW, Archer C, Craik DJ, Nielsen LK. Proteome analysis of the hyaluronic acid-producing bacterium, *Streptococcus zooepidemicus*. Proteome Sci. 2009;28(7):13. https://doi.org/10.1186/1477-5956-7-13.
32. Gedikli S, Güngör G, Toptaş Y, Sezgin DE, Demirbilek M, Yazıhan N, Çelik PA, Denkbaş EB, Bütün V, Çabuk A. Optimization of hyaluronic acid production and its cytotoxicity and degradability characteristics. Prep Biochem Biotechnol. 2018; 48: 610–18. https://doi.org/10.1080/10826068. 2018. 1476885.
33. Shah MV, Badle SS, Ramachand. Hyaluronic acid production and molecular weight improvement by redirection of carbon flux towards its biosynthesis pathway. Biochem Eng J. 2013;80(15):53–60. https://doi.org/10.1016/j.ijbiomac.2020.02.214.
34. Zhang Y, Luo K, Zhao Q, Qi Z, Nielsen LK, Liu H. Genetic and biochemical characterization of genes involved in hyaluronic acid synthesis in *Streptococcus zooepidemicus*. Appl Microbiol Biotechnol. Apr; 2016;100(8):3611–20. https://doi.org/10.1007/s00253-016-7286-1.
35. Zhou S, Ding R, Chen J. Obtaining a panel of Cascade Promoter-5′-UTR complexes in *Escherichia coli*. ACS Synth Biol. 2017;6(6):1065–75.
36. Song Y, Nikoloff JM, Fu G. Promoter screening from *B.subtilis* in various conditions Hunting for Synthetic Biology and Industrial Applications. PLoS ONE. 2016;11(7):e0158447.
37. Sullivan MJ, Ulett GC. Stable expression of modified green fluorescent protein in group B streptococci to enable visualization in experimental systems. Appl Environ Microb. 2018;84(18):e01262–18.
38. Zhang XZ, Wang M, Li TJ, Fu LX, Cao W, Liu H. Construction of efficient *Streptococcus zooepidemicus* strains for hyaluoronic acid production based on identification of key genes involved in sucrose metabolism. AMB Expr. 2016;6(1):121–30. https://doi.org/10.1186/s13568-016-0296-7.
39. Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol. 2005;68:705–17. https://doi.org/10.1007/s00253-005-0107-6.
40. Prasad SB, Jayaraman G, Ramachandran KB. Hyaluronic acidproduction is enhanced by the additional co-expression of UDP glucose pyrophosphorylase in *Lactococcus lactis*. Appl Microbiol Biotechnol. 2010;86(1):273–83. https://doi.org/10.1007/s00253-009-2293-0.
41. Widner B, Behr R, Von Dollen S, Tang M, Heu T, Sloma A, Sternberg D, DeAngelis PL, Weigel PH, Brown S. Hyaluronic acid production in *Bacillus subtilis*. Appl Environ Microbiol. 2005;71(7):3747–52. https://doi.org/10.1128/AEM.71.7.3747-3752.2005.
42. Chahuki FF, Aminzadeh S, Jafarian V, Tabandeh F, Khodabandeh M. Hyaluronic acid production enhancement via genetically modification and culture medium optimization in Lactobacillus acidophilus. Int J Biol Macromol. 2019; 121 870–81, 10.1016/ j.ijbiomac. 2018.10.112.
43. Rangaswamy V, Jain D. An efficient process for production and purification of hyaluronic acid from *Streptococcus equi subsp. zooepidemicus*. Biotechnol Lett. 2008;30:493–6. https://doi.org/10.1007/s10529-007-9562-8.
44. Pan NC, Vignoli JA, Baldo C, Pereira HCB, Silva RSSF, Celligoi MAPC. Effect of fermentation conditions on the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920. Acta Sci - Biol Sci. 2015;37:411–7. https://doi.org/10.4025/actascibiolsci.v37i4.28176.
45. Jagannath S, Ramachandran KB. Influence of competing metabolic processes on the molecular weight of hyaluronic acid synthesized by *Streptococcus zooepidemicus*[J]. Biochem Eng J. 2010;48(2):148–58. https://doi.org/10.1016/j.carbpol.2023.120809.
46. Kumari K, Tlapak-simmons V, Baggenstoss B. The streptococcal hyaluronan synthases are inhibited by sulfhydryl-modifying reagents,but conserved cysteine residues are not essential for enzyme function[J]. J Biol Chem. 2002;277(16):943–51. 10.1074/ jbc. M110638200.
47. Stern R, Jedrzejas MJ. Hyaluronidases: their genomics, structures, and mechanisms of action. Chem Rev. 2006;106(3):818–39. https://doi.org/10.1021/cr050247k.
48. Kumari K, Weigel PH. Molecular cloning, expression, and characterization of the authentic hyaluronan synthase from group C *Streptococcus equisimilis*. J Biol Chem. 1997;272(51):32539–46. https://doi.org/10.1074/jbc.272.51.32539.
49. Heldermon C, DeAngelis PL, Weigel PH. Topological organization of the hyaluronan synthase from *Streptococcus pyogenes*. J Biol Chem. 2001;276(3):2037–46. https://doi.org/10.1074/jbc.M002276200.
50. Baggenstoss BA, Harris EN, Washburn JL, Medina AP, Nguyen L, Weigel PH. Hyaluronan synthase control of synthesis rate and hyaluronan product size are independent functions differentially affected by mutations in a conserved tandem B-X7-B motif. Glycobiology. 2017;27(2):154–64. https://doi.org/10.1093/glycob/cww089.

Publisher's note