

A Biosafe *Bacillus subtilis* 168 Engineered for Hyaluronic Acid Biosynthesis

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Abstract

Background and Objective: Hyaluronic acid is a high-value biopolymer widely used in the food, cosmetic, therapeutic and pharmaceutical industries. This study aimed to establish a biosafe “Generally Recognized as Safe” platform for hyaluronic acid biosynthesis via engineering *Bacillus subtilis* 168.

Material and Methods: The hyaluronan synthase gene from *Streptococcus dysgalactiae* and *tuaD* homolog NX02_04625 from *Sphingomonas sanxanigenens* were selected based on their reported catalytic performance and codon-optimized for *Bacillus subtilis*. The complete pHT01 vector construct harboring the two genes was commercially synthesized and sequence-verified. A single-gene plasmid was generated and the two plasmids were introduced into *Bacillus subtilis* 168 via electroporation. Hyaluronic acid production was quantified using cetyltrimethylammonium bromide assay, structurally validated by Fourier transform infrared spectroscopy and characterized by size-exclusion chromatography.

Results and Conclusion: The dual-gene strain produced 1.20 ± 0.03 g l⁻¹ hyaluronic acid, achieving a 2.4-fold increase relative to the single-gene strain and showed a trimodal molecular-weight profile dominated by high-molecular-weight polymers (> 1.18 MDa, 43.45%), accompanied by medium-range species (~0.99 MDa, 24.69%) and low-molecular-weight-oligomers (~55 kDa, 31.86%). Collectively, these findings highlight the efficiency and biosafety of the engineered production platform and its potential for food-grade uses, supported by the predominance of high-molecular-weight hyaluronic acid with favorable gel-like characteristics.

Keywords: *Bacillus subtilis* 168, Hyaluronan synthase gene, Hyaluronic acid production, Metabolic engineering, Strain development, UDP-glucose dehydrogenase

What is “already known” on this topic:

- Industrial hyaluronic acid production mainly relies on pathogenic *Streptococcus*, raising biosafety concerns.
- *Bacillus subtilis* is a safer host with generally recognized as safe status, but its hyaluronic acid production efficiency remains limited.
- UDP-GlcUA availability is a key metabolic bottleneck in hyaluronic acid biosynthesis in *Bacillus subtilis*.

What this article adds:

- The engineered dual-gene *Bacillus subtilis* strain achieved higher hyaluronic acid production than the single-gene strain by addressing the metabolic bottleneck through co-expression of *hasA* and *tuaD* homologs.
- Fourier-transform infrared spectroscopy and size-exclusion chromatography analyses verified hyaluronic acid identity and the predominance of high-molecular-weight fractions, supporting the effectiveness of this co-expression strategy.

1. Introduction

Hyaluronic acid (HA) is a high-value, linear glycosaminoglycan renowned for its exceptional biocompatibility, viscoelasticity and hygroscopic characteristics [1]. As established in recent literature [2], HA is structurally composed of repeating disaccharide units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc). The HA is a ubiquitous component of the extracellular matrix in vertebrates; where, it regulates cell proliferation, tissue hydration, inflammation and wound repair [3]. Due to its shear-thinning behavior and high moisture retention, HA has become essential in biomedicine (e.g. dermal fillers and drug delivery), cosmetics (e.g. anti-aging formulations) and regenerative medicine [4, 5].

The unique physicochemical characteristics of HA, including its molecular weight-dependent biological functions, support its clinical versatility [1]. High-molecular-weight (HMW) HA is generally associated with tissue homeostasis and restraint of cell proliferation/migration, whereas HA fragmentation into low-molecular-weight (LMW) species is linked to inflammatory and promigratory signaling [6]. The LMW-HA can enhance endothelial angiogenic responses and migration *in vitro* [7], supporting the use of tailored HA preparations for various therapeutic uses.

In comparative studies using mouse models of persistent inflammation, immunosuppression and catabolism syndrome, low (3 kDa), medium (100 kDa) and HMW (1600 kDa) HA were assessed at 30 mg kg⁻¹, revealing distinct biological responses as 1600 kDa HA significantly promoted recovery of intestinal structure and was associated with improved immune recovery while 100 kDa HA induced stronger inflammatory cell infiltration after prolonged treatment and 3 kDa HA showed comparatively milder effects [8].

The HA is a promising safe additive for functional foods. In yogurt, HA at 180, 350 and 1280 kDa showed that 350 kDa improved pH, texture and sensory quality most effectively; 1280 kDa increased stability but caused whey syneresis and 180 kDa decreased stability [9]. For sweetness at pH 4.0, HA at 100, 400 and 1090 kDa extended sweet taste duration, with 100 kDa showing the strongest longest-lasting effect via enhanced sucralose transport. Advances in sustainable production support broader uses, with HMW-HA critical for gel-like texture, stability and viscosity [10]. In meats, a 0.9% HA coating has preserved the freshness of crucian carp for 24 d [11]. The HA has formed stable complexes with fish gelatin, improving its emulsifying characteristics [12]. Products such as Cenovis HA beverage [13] and rooster comb extract in dairy products [14] demonstrate the increasing use of food-grade HA. The HA strength, biodegradability and biocompatibility include

excellent potential for edible packaging. Its versatility in forming gels and complexes with neutral taste and color makes it a promising material for food coatings and edible packaging materials [15–17].

Conventional HA production methods face critical limitations. Fermentation using pathogenic *Streptococcus* strains (*S. zooepidemicus*) increases biosafety concerns and complicates the downstream processing [18, 19]. The relatively low yield of natural HA producers makes conventional production economically and industrially less feasible. This limitation has accelerated efforts to develop genetically engineered generally recognized as safe (GRAS) microbial platforms for sustainable HA synthesis [20, 21]. In recent years, *Bacillus subtilis* (*B. subtilis*) has attracted significant attentions as a microbial cell factory for the biosynthesis of valuable biomolecules and biopolymers [22–25]. Well-characterized genome, efficient protein secretion system and strong metabolic capacity has made *B. subtilis* a particularly attractive chassis for metabolic engineering and synthetic biology uses [22, 26].

Several studies have demonstrated the feasibility of engineering *B. subtilis* to produce heterologous biomolecules, highlighting its potential as a safe and scalable platform for industrial biotechnology [23, 27]. Regarding its non-pathogenic nature, endotoxin-free status and established use in industrial biotechnology, this bacterium has been engineered to produce enzymes, polysaccharides and vitamins [23, 24, 28, 29]. In addition, engineered *B. subtilis* has been used for the production of industrial biopolymers such as poly- γ -glutamic acid, further highlighting its potential as a microbial cell factory [30]. However, engineering *B. subtilis* for HA production presents specific genetic and metabolic challenges. Unlike natural HA producing organisms such as *Streptococcus* species, *B. subtilis* lacks a native hyaluronan synthase gene responsible for the polymerization of HA from UDP sugar precursors. Therefore, reconstruction of the HA biosynthetic pathway needs heterologous expression of a functional hyaluronan synthase and *B. subtilis* only carries precursor-related genes such as *tuaD* (UDP-glucose dehydrogenase) [21, 22, 31]. In addition, efficient HA synthesis depends on a balanced intracellular supply of key precursors. Limitations in precursor availability and metabolic flux distribution can restrict HA yield, making pathway optimization and targeted metabolic engineering essential for achieving efficient HA production in this host [22, 24]. In addition to heterologous expression of HA synthase, efficient HA production in *B. subtilis* needs precise metabolic rewiring. This involves redirecting the carbon flux by modulating the competing pathways such as glycolysis and the pentose phosphate pathway while



maintaining cellular energy balance. For example, complete inactivation of the *pfkA* gene is lethal, indicating that these pathways must be carefully fine-tuned rather than fully disrupted [22]. Recent developments in strain engineering and bioprocess optimization with advances in enzyme/pathway engineering have enabled the identification of improved UDP-sugar pathway enzymes and hyaluronan synthases that enhance HA biosynthesis in non-pathogenic hosts [21, 22, 32–34].

In this study, a *B. subtilis* strain was developed that was capable of efficient HA production through heterologous expression of the hyaluronan synthase gene, which is absent in the native genome and NX02_04625, a functional homolog of *tuaD* that enhances the supply of UDP-GlcUA precursors [22, 24]. Single and dual-gene expression systems were also constructed and compared. By introducing a novel genetic combination of hyaluronan synthase gene with an additional *tuaD* homolog from another bacterial source with favorable functional traits, this approach establishes a novel expression platform that addresses biosafety concerns associated with pathogenic production strains and provides a promising foundation for scalable HA biosynthesis. Unlike previous studies that overexpressed the native *tuaD* or used various hyaluronan synthase sources, this study co-expressed a codon-optimized hyaluronan synthase from *S. dysgalactiae* with a high-performance *tuaD* homolog (NX02_04625) from *Sphingomonas sanxanigenens*, a combination not previously reported in *B. subtilis*. This approach potentially improves critical metabolic difficulties identified in previous studies and contributes to the advancement of safe and efficient microbial platforms for HA production.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Escherichia coli (*E. coli*) DH5 α ATCC 53868 used for plasmid propagation and *B. subtilis* 168 ATCC 23857 used as the expression host for HA production were provided by the National Institute of Genetic Engineering and Biotechnology. Cultures were grown in Luria-Bertani (LB) medium (10 g l⁻¹ peptone, Merck, Germany; 5 g l⁻¹ yeast extract, Merck, Germany; 10 g l⁻¹ NaCl, Merck, Germany). Precultures were prepared by inoculating a single colony into 5 ml of LB medium and incubating overnight at 37 °C with agitation at 180 rpm. For plasmid maintenance, appropriate antibiotics were added to the medium, including ampicillin (Sigma-Aldrich, USA) at a final concentration of 100 μ g ml⁻¹ for *E. coli* and chloramphenicol (Sigma-Aldrich, USA) at a final concentration of 10 μ g ml⁻¹ for *B. subtilis*. For production experiments, the overnight preculture was transferred into fresh medium at 1% (v/v). The selected production medium contained sucrose (30 g

l⁻¹; Merck, Germany), yeast extract (10 g l⁻¹; Merck, Germany), soybean peptone (5 g l⁻¹; Merck, Germany), ammonium sulfate (1 g l⁻¹; Merck, Germany), K₂HPO₄ (9.15 g l⁻¹; Merck, Germany), KH₂PO₄ (3 g l⁻¹; Merck, Germany) and trisodium citrate (1 g l⁻¹; Merck, Germany) [22].

Gene expression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM (Thermo Fisher Scientific, USA) at the beginning of cultivation and the cultures were incubated at 30 °C with agitation at 180 rpm. Samples were collected at designated time points (24, 48 and 72 h after inoculation of the production culture, depending on the experiment) and centrifuged at 2400 \times g for 5 min to collect cell-free supernatants, which were used for further analyses.

2.2. Gene selection and vector design

To confer HA biosynthetic capability to *B. subtilis* 168, hyaluronan synthase gene from *S. dysgalactiae* ATCC12394 was selected based on its catalytic performance reported in the Braunschweig Enzyme Database. The gene encodes a hyaluronan synthase used for construct assembly and experimental procedures. To further enhance HA production, NX02_04625 from *S. sanxanigenens* DSM19645 was coexpressed, which was a *tuaD* homolog with favorable kinetic characteristics.

The two genes were codon-optimized for *B. subtilis* (designed using SnapGene v.3.2.1; GSL Biotech, USA). These were then inserted into the shuttle vector pHT01 under the IPTG-inducible promoter P_{grac01}, forming a single transcriptional unit with an intergenic ribosome binding site to enable independent translation. A schematic representation of the dual-gene construct (pHT01-hyaluronan synthase gene-NX02_04625), designed for HA production in *B. subtilis* 168 (Figure 1), synthesized and sequence-verified by Gene Universal, USA, through its official representative of Pars Biotek, Iran. A single-gene control plasmid was constructed *in house*.

2.3. Cloning procedures and construct validation

The dual-gene plasmid, hereafter referred to as PST, was reconstituted and diluted to a working concentration. Chemically competent *E. coli* DH5 α cells were transformed using the standard heat-shock method with minor modifications. Transformed cells were plated on LB agar containing ampicillin [35]. To excise the NX02_04625 region, the PST was digested with the *Xba*I restriction enzyme (Thermo Fisher Scientific, USA; lot no. 01258845), resolved on agarose gel and the PS was excised and purified using FavorPrep gel/PCR purification mini kit (Favorgen Biotech, Taiwan; cat. no. FAGCK001) according to the manufacturer's instructions. The purified backbone-hyaluronan synthase gene, referred to as PS, was ligated using T4 DNA ligase (Thermo Fisher Scientific, USA; lot no. 00024630).



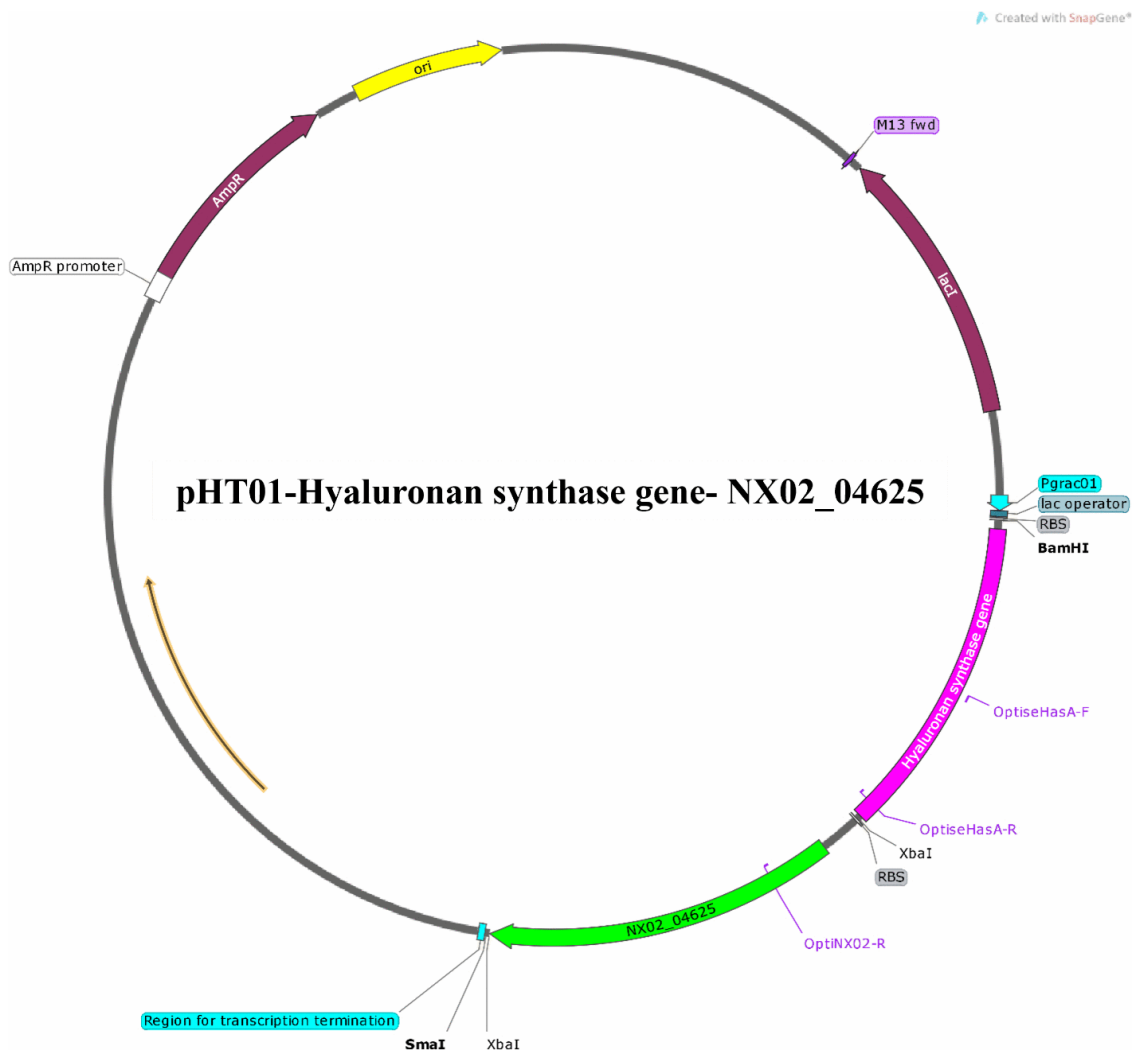


Figure 1. Schematic plasmid map of the dual-gene construct designed for hyaluronic acid biosynthesis in *Bacillus subtilis* 168. The construct included the isopropyl β -D-1-thiogalactopyranoside-inducible Pgrac01 promoter, lac operator, ribosome-binding site, codon-optimized hyaluronan synthase gene and NX02_04625 and transcription terminators. RBS, Ribosome-binding site; OptiseHasA-F, forward primer for hyaluronan synthase gene; OptiseHasA-R, reverse primer for hyaluronan synthase gene; OptiNX02-R, reverse primer for NX02_04625. Restriction sites used for cloning and excision included *Bam*HI, *Sma*I and *Xba*I.

Ligation products were transformed into *E. coli* DH5 α and plated on ampicillin LB agar. Plasmid DNA was extracted using plasmid DNA extraction mini kit (Favorgen Biotech, Taiwan; cat. no. FAPDE050) according to the manufacturer's instructions. Construct sizes were verified using gel electrophoresis.

To prepare 0.7% (w/v) agarose gels, 0.7 g of agarose was dissolved in 100 ml of 0.7 \times tris-borate-EDTA (TBE) buffer. The mixture was heated using microwave. After cooling down, the gel was poured into a casting tray and a comb was inserted to form the wells. After solidification, the gel was transferred into an electrophoresis tank filled with 0.7 \times TBE buffer. The DNA samples were loaded and separated at a constant voltage of 5–8 V cm $^{-1}$ until adequate migration was

achieved. Band sizes were determined by comparison with 1-kb plus DNA ladder (SinaClon, Iran; cat. no. SL7052).

2.4. Transformation into *Bacillus subtilis* 168 and validation

The minimum inhibitory concentration (MIC) of chloramphenicol for *B. subtilis* 168 was assessed as 10 μ g ml $^{-1}$. Electrocompetent *B. subtilis* cells were transformed with PS and PST constructs via electroporation [36] and selected on LB-chloramphenicol plates. Colonies were validated using colony PCR and gene-specific primers synthesized by Genfanavar, Iran. Colony PCR was carried out on *B. subtilis* transformants to verify insertion of recombinant constructs.



2.5. Hyaluronic acid purification

From the media [27, 37], only the modified Westbrook formulation [22] supported HA synthesis. Culture supernatants from engineered *B. subtilis* were clarified by centrifugation and subjected to sequential trichloroacetic acid (Merck, Germany) and ethanol (Merck, Germany) precipitation. For trichloroacetic acid precipitation, 100 μ l of 100% (w/v) trichloroacetic acid were added to 1 ml of clarified supernatant, followed by incubation on ice for 30 min and centrifugation at 16,200 \times g for 15 min at 4 °C. The resulting supernatant was mixed with 2 ml of cold ethanol (absolute), transferred to -70 °C for 2 h and centrifuged at 4,400 \times g for 20 min at 4 °C. The pellet was washed with 1 ml of cold ethanol (absolute), incubated at -20 °C for 1 h and recentrifuged. After air-drying, the HA pellet was dissolved in 1 ml of deionized water and incubated at 45 °C with agitation until fully solubilized. Samples were stored at 4 °C until further analysis.

2.6. Cetyltrimethylammonium bromide turbidimetric method

The HA levels were quantified using cetyltrimethylammonium bromide turbidimetric assay. Samples were mixed with acetate buffer (0.2 M, pH 6; Merck, Germany) and cetyltrimethylammonium bromide reagent (2.5% w/v in 0.5 M NaOH; Merck, Germany) and absorbance was measured at 540 nm. A standard curve prepared from commercial HA (Sigma-Aldrich, USA; lot no. BCBN4845V) was used for the quantification, ensuring a consistent linear relationship between absorbance and HA concentration within the assay working range [38].

2.7. Fourier-transform infrared analysis

Fourier-transform infrared (FTIR) spectroscopy was used to verify the structural identity of the purified HA. Spectra were recorded in the range of 4000–400 cm^{-1} using standard scanning parameters on the Bruker FTIR spectrometer (Bruker, USA). Characteristic peaks corresponding to hydroxyl, carboxyl and amide groups were assessed.

2.8. Molecular weight determination by size-exclusion chromatography

Size-exclusion chromatography separates molecules based on their hydrodynamic size, which depends on molecular weight and molecular conformation in solution. This technique is historically known as gel filtration or gel permeation chromatography (GPC) [39]. The GPC was carried out to assess molecular weight distribution of the purified HA samples. The system (Waters, USA) was equipped with two PL aquagel-OH 8 μ m mixed-H columns (300 \times 7.5 mm each, separation range 6,000–10⁷ g mol⁻¹; Agilent Technologies, USA) and a refractive index detector (Waters 2414, Waters, USA). Deionized water was used as the mobile phase at a flow rate of 1.0 ml min⁻¹ and the

column temperature was set at 35 °C. Calibration was carried out using polyethylene glycol standards (Agilent Technologies, USA), ranging from 1.03 \times 10³ to 1.18 \times 10⁶ g mol⁻¹. All samples were filtered through 0.45- μ m membrane filters (Merck Millipore, USA) prior to injection.

2.9. Statistical analysis

All experiments were carried out with at least two biological replicates and results were expressed as mean \pm SD (standard deviation). Normality of data was assessed graphically. Statistical comparisons were carried out using Microsoft Excel v.16.79, USA, and GraphPad Prism v.9.5.0, USA. The HA concentrations, calculated based on optical density values and validated standard curve, were used for statistical analyses. For comparison between the wild-type and PST strains, Welch's two-tailed t-test was used to account for unequal variances. One-tailed t-test was used to compare HA production between the PS and PST strains. Paired t-test was used for temporal comparisons (24 against 48 h, 48 against 72 h) within each strain. A *p*-value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Construction of the single-gene construct

Polymerase chain reaction analysis of the PST verified the expected amplicon sizes for the two target regions, validating construct integrity. Transformation into *E. coli* DH5 α yielded robust colony formation on selective medium and plasmid extraction produced DNA of the expected size, verifying plasmid stability. Digestion with *Xba*I generated fragments of the anticipated sizes, enabling recovery and successful assembly of the PS construct.

3.2. Verification of single-gene construct via plasmid size comparison

Size analysis of the plasmid preparations showed a clear distinction between the two constructs. Figure 2 illustrates the agarose gel electrophoresis of the PS and PST constructs. The upward shift observed for the PST variant verified the expected size increase, consistent with the presence of NX02_04625 in PST and its successful excision during generation of the PS construct.

3.3. Introduction of single and dual-gene constructs into *Bacillus subtilis* 168

The PS and PST constructs were successfully established in *B. subtilis* 168, as evidenced by the formation of discrete and stable colonies on selective medium. Colony-level analysis verified the expected inserts in *B. subtilis* 168 transformants. The PS and PST transformants respectively produced amplicons, indicating the presence of PS and PST constructs and verifying successful uptake and stable maintenance in the host strain.



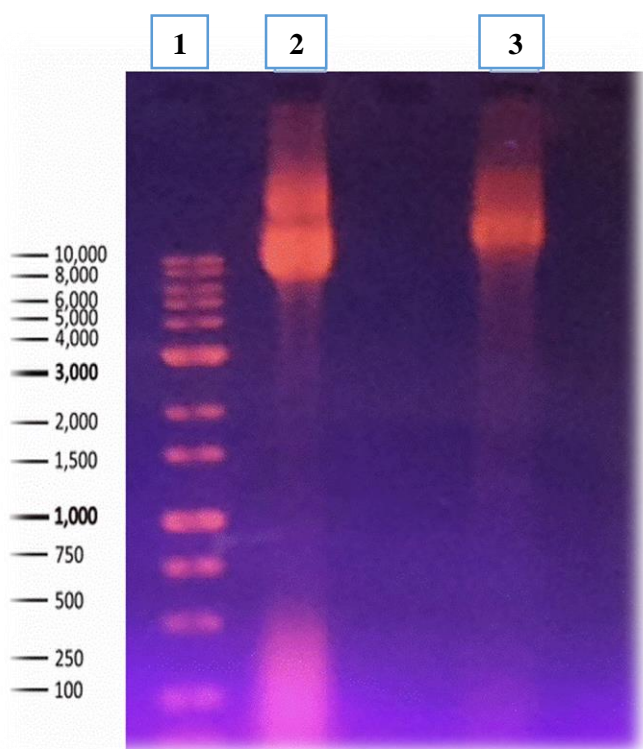


Figure 2. Agarose gel electrophoresis comparing plasmid sizes of the single and dual-gene constructs. Lane 1, 1-kb plus DNA ladder; Lane 2, single-gene construct; and Lane 3, dual-gene construct. The smaller size of the single-gene construct verified successful excision of the NX02_04625 segment.

3.4. Initial assessment of hyaluronic acid yield in wild-type against dual-gene recombinant *Bacillus subtilis*

The comparison between the wild-type and PST-harboring *B. subtilis* 168 strains in LB medium revealed clear evidence of HA production. Figure 3 illustrates the HA concentrations produced by these two strains at 24 and 48 h. The PST recombinant produced 0.22 ± 0.03 and 0.31 ± 0.02 g l⁻¹ HA at 24 and 48 h respectively, whereas only a negligible signal was observed in the wild-type strain, likely due to background turbidity in the assay. The differences between the two strains were statistically significant at the two sampling times, indicating the HA biosynthetic capacity conferred by the PST construct.

3.5. Comparison of hyaluronic acid production in single and dual-gene-engineered *Bacillus subtilis* strains

Modified Westbrook fermentation medium was used for comparative evaluation of the PS and PST strains [22]. Figure 4 shows the HA concentrations produced by PS and PST at 24, 48 and 72 h post-induction. In the PST, HA levels reached 1.20 ± 0.03 g l⁻¹ at 48 h and 1.14 ± 0.07 g l⁻¹ at 72 h with no significant difference between the two time points ($p > 0.05$). The PS produced 0.49 ± 0.07 g l⁻¹ at 48 h and

0.50 ± 0.13 g l⁻¹ at 72 h, showing no significant temporal change ($p > 0.05$). At the two sampling times, HA production in the PST strain was higher than that in the PS strain ($p = 0.064$), suggesting improved biosynthetic performance of the dual-gene construct.

The PST construct demonstrated an advantage over the PS construct, achieving a HA yield of 1.20 ± 0.03 g l⁻¹ in shake-flasks compared to 0.50 ± 0.13 g l⁻¹, a 2.4-fold increase. This clear difference highlighted critical difficulties in the precursor supply chain, specifically the conversion of UDP-glucose to UDP-GlcUA, which apparently improved by the expression of NX02_04625. This finding aligned with and reinforced the metabolic engineering principle established by Westbrook et al. [22], who identified the increase of the UDP-GlcUA pool via the native *tuaD* gene as the most impactful intervention, boosting the HA titer 5.6-fold in their *B. subtilis* system.

A broader comparison of HA yield and molecular weight reported in previous studies provided useful context for interpreting the current results. The *B. subtilis* systems produce approximately 1–2 g l⁻¹ HA in shake-flask cultures. Under fed-batch fermentation conditions, *Corynebacterium glutamicum* achieved up to 28.7 g l⁻¹ (0.21 MDa) [40] and 21.6 g l⁻¹ (1.28 MDa) [41]. Studies on *B. subtilis* in shake-flask systems have reported yields nearly 1.2 g l⁻¹ [22, 24], with molecular weights ranging from 1.5–2.0 MDa [22]. Zhang et al. reported a yield of 1.8 g l⁻¹ under similar conditions [37]. It is noteworthy that the reported HA yields depended strongly on cultivation conditions; fed-batch bioreactor systems generally achieve higher titers than shake-flask cultures. Therefore, the values should be interpreted as approximate comparisons between various production platforms, rather than direct performance equivalents.

The core of our engineering strategy was the rational reconstruction and enhancement of the heterologous HA biosynthetic pathway, enabling a final HA yield of 1.20 g l⁻¹ in shake-flasks. Native *B. subtilis* possesses the genetic machinery for UDP-N-acetylglucosamine (UDP-GlcNAc) synthesis but lacks the hyaluronan synthase gene and shows limited flux toward the second precursor of UDP-GlcUA [22, 31]. The present approach involved the heterologous coexpression of hyaluronan synthase gene from *S. dysgalactiae* and a high-performance *tuaD* homolog (NX02_04625) from *S. sanxanigenens*, selected for its favorable enzymatic kinetics from the Braunschweig Enzyme Database.

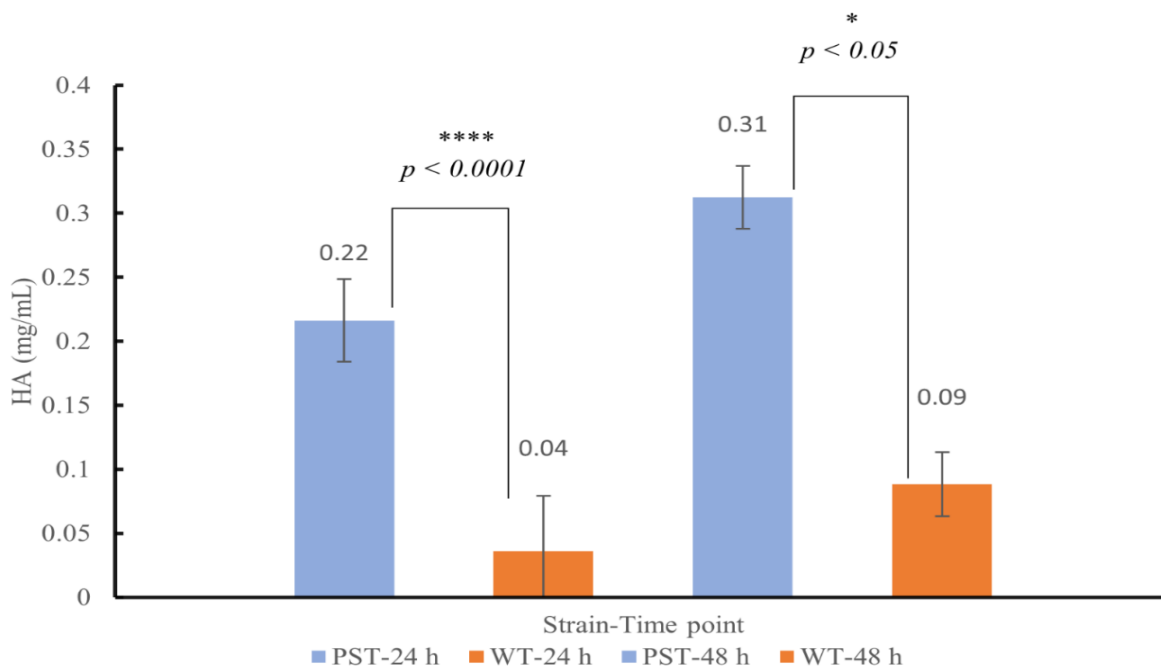


Figure 3. Hyaluronic acid production in wild-type and dual-gene recombinant *Bacillus subtilis* 168 strains at 24 and 48 h. The x-axis represents time post-induction (h) and the y-axis indicates hyaluronic acid concentration (g l^{-1}). Bars represent mean \pm SD ($n = 3$). The PST at 24 h (0.22 g l^{-1}); PST at 48 h (0.31 g l^{-1}); wild-type at 24 h (0.04 g l^{-1}); and wild-type at 48 h (0.09 g l^{-1}). Asterisks indicate statistical significance ($*p < 0.05$, $****p < 0.0001$). PST, Dual-gene recombinant *Bacillus subtilis* 168 strains; and WT, wild-type strain.

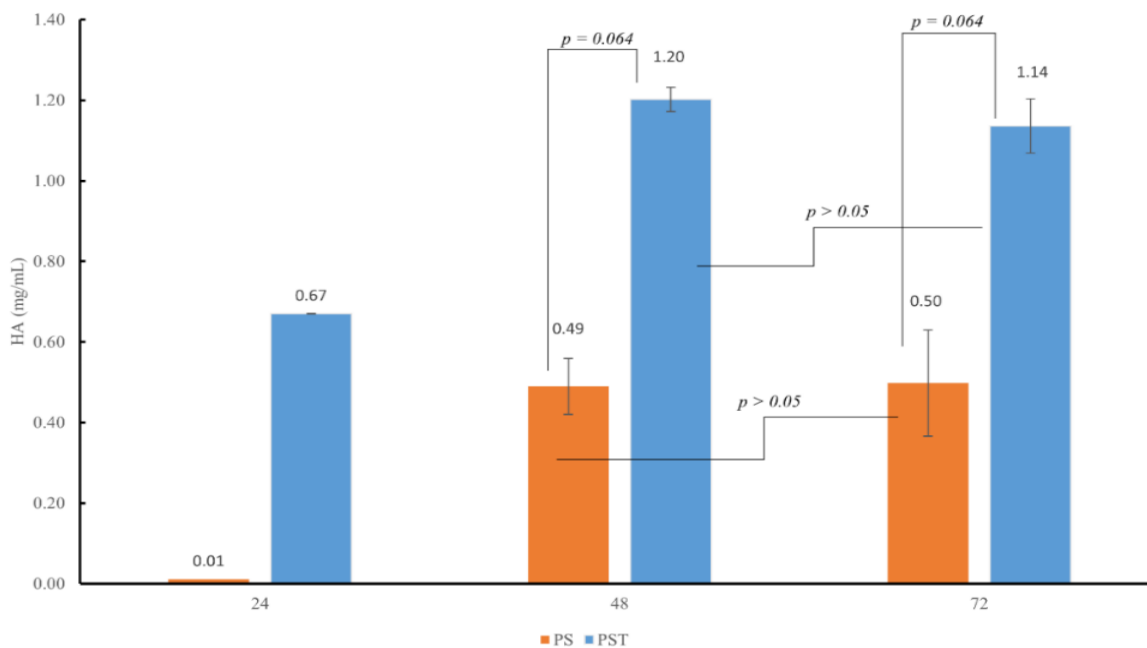


Figure 4. Hyaluronic acid production in recombinant *Bacillus subtilis* 168 strains carrying single and dual-gene constructs at 24, 48 and 72 h post-induction. The x-axis represents time post-induction (h) and the y-axis indicates hyaluronic acid concentration (g l^{-1}). Bars represent mean \pm SD ($n = 2$). No significant difference was observed between 48 and 72 h within each strain ($p > 0.05$, paired t-test). At the two time points, hyaluronic acid production in the dual-gene-engineered strain was higher than that in the single-gene-engineered strain, showing a consistent trend ($p = 0.064$, one-tailed t-test). PST, Dual-gene recombinant *Bacillus subtilis* 168 strains; and PS, single-gene recombinant *Bacillus subtilis* 168 strains.



3.6. Fourier-transform infrared analysis of produced hyaluronic acid

Figure 5 shows the FTIR spectrum of purified HA produced by engineered *B. subtilis* 168 carrying the PST plasmid. This spectrum showed the characteristic absorption bands of HA, including O–H stretch (3415 cm^{-1}), C–H stretch (2934 cm^{-1}), asymmetric and symmetric COO^- stretches (1683 and 1459 cm^{-1}) and C–O–C vibration at 1097 cm^{-1} . The current characterization methods supported the biosynthesis of HA. The FTIR spectroscopy showed absorption bands attributable to key functional groups (O–

H, COO^- and C–O–C) consistent with previously reported HA spectra [42]. This was consistent with functional heterologous expression of the hyaluronan synthase gene, a critical step toward biomedical applicability [43].

3.7. Molecular weight distribution by size-exclusion chromatography

Figure 6 illustrates results of size-exclusion chromatography analysis of purified HA produced by engineered *B. subtilis* 168 carrying the PST plasmid. This revealed a trimodal distribution.

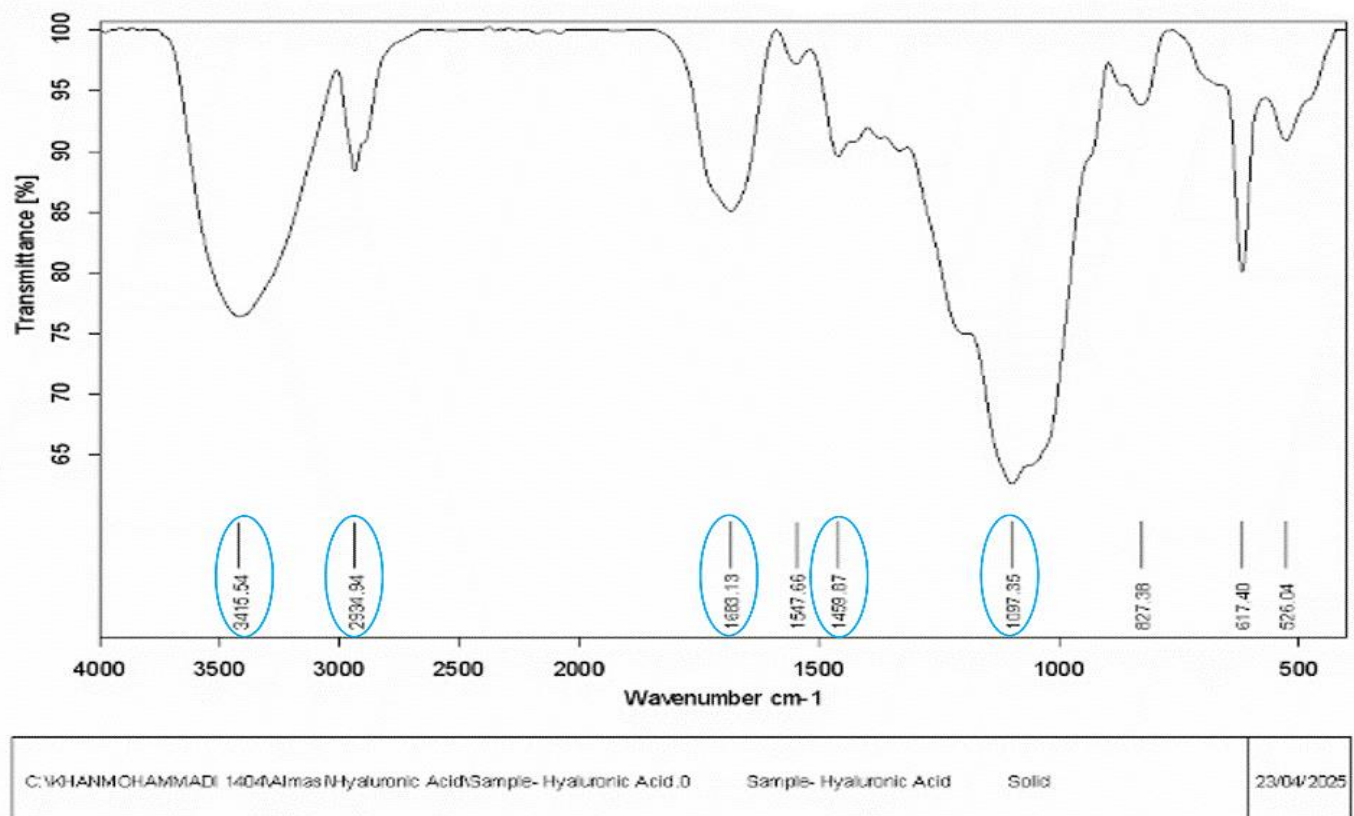


Figure 5. Fourier-transform infrared spectrum of hyaluronic acid produced by the engineered *Bacillus subtilis* 168 carrying the dual-gene construct. Characteristic absorption bands corresponding to O–H stretching ($\sim 3415\text{ cm}^{-1}$), C–H stretching ($\sim 2934\text{ cm}^{-1}$), a band in the carbonyl region ($\sim 1683\text{ cm}^{-1}$), COO^- stretching ($\sim 1459\text{ cm}^{-1}$) and C–O–C stretching ($\sim 1097\text{ cm}^{-1}$) were observed.

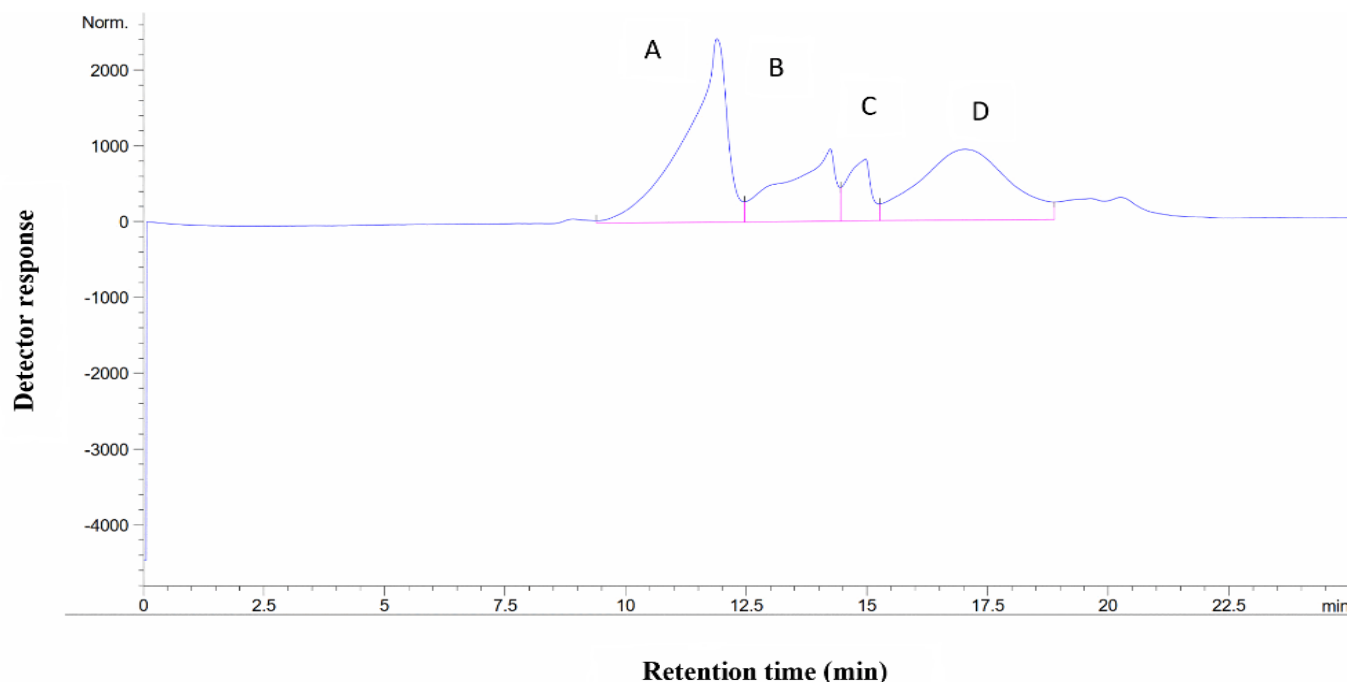


Figure 6. Size-exclusion chromatography chromatogram of hyaluronic acid produced by the engineered *Bacillus subtilis* 168 carrying a dual-gene construct, showing (A) high-molecular-weight native hyaluronic acid, (B) fragmented hyaluronic acid, and (C) oligomers. Dashed lines denote calibration limits. Peak 1 (retention time = 11.884 min), 43.45% of total area, assigned to high-molecular-weight native hyaluronic acid ($> 1.18 \times 10^6$ g mol⁻¹); Peaks 2 and 3 (retention time = 14.229–14.956 min), 24.69% of total area, assigned to moderately fragmented hyaluronic acid (~ 0.99 MDa); Peak 4 (retention time = 17.030 min), 31.86% of total area, assigned to low-molecular-weight hyaluronic acid oligomers (~ 0.055 MDa).

3.7.1. Molecular weight parameters

Quantitative MW data for fractions within the calibration range are reported in Table 1. The HMW-HA (Peak 1) exceeded the column calibration range; its MW was estimated as $> 1.18 \times 10^6$ g mol⁻¹ but needed absolute quantification. The GPC analysis revealed a heterogeneous molecular weight distribution, which was typical for microbial HA production due to the presence of endogenous hydrolases or shear stress [44, 45]. The trimodal distribution indicated significant heterogeneity. The dominant HMW fraction (43.45%) aligned with native HA intrinsic chain length, while the mid and LMW fractions (56.55% combined) suggested partial hydrolysis during processing. The narrow PDI (1.20) of fragmented HA suggested controlled degradation, whereas the broader PDI (1.34) of oligomers reflected heterogeneous chain cleavage. In the present study, the engineered *B. subtilis* strain produced HA with a dominant molecular weight exceeding 1.18 MDa. The HMW-HA (> 1.18 MDa) is highly valued in medical and food uses [46, 47]. While this heterogeneity might need further purification for specific uses, it presented an opportunity as low and medium-MW-HA fractions include their own unique therapeutic and food grade uses [48–50]. The average molecular weight of the main fragmented fraction was calculated at 9.88×10^5 g mol⁻¹, which was within the highly desirable range for commercial uses [51].

Table 1. Molecular weight distribution of validated HA fractions.

| Fraction | % Area | M _n (g mol ⁻¹) | M _w (g mol ⁻¹) | PDI |
|---------------|--------|--|--|------|
| Fragmented HA | 24.69 | 8.26×10^5 | 9.88×10^5 | 1.20 |
| Oligomers | 31.86 | 4.12×10^4 | 5.54×10^4 | 1.34 |

M_n: Number-average molecular weight; M_w: Weight-average molecular weight; PDI: Polydispersity Index (M_w/M_n).

A previous study reported that HMW-HA increased the hardness of ginkgo seed protein isolate gels and improved *in vitro* digestion by up to 70%. Gel texture and digestibility could be controlled by adjusting pH and HA molecular weight, likely due to physical entanglement between HA and protein molecules that restricted their movement and stabilized the gel network [52]. The HMW-HA enhanced texture (via stronger gel networks) and nutrition-related attributes such as digestibility. Its mucoadhesive characteristics prolonged the contact time of substances with taste receptors, aiding in the perception of saltiness [50].

The escalating demand for HA in the food, pharmaceutical, biomedical and cosmetic industries necessitates a paradigm shift away from traditional, limited production methods. While extraction from animal tissues increases concerns about pathogen contamination and batch-to-batch variability and fermentation using pathogenic *Streptococcus* strains introduces significant



biosafety and regulatory hurdles, the development of efficient GRAS microbial platforms has become a central focus of metabolic engineering. The advantage of the current *B. subtilis*-based system includes its combination of a GRAS status, high secretory capacity, well-known genetics and absence of endotoxins, which may facilitate safer development and regulatory consideration for food and biomedical uses, compared to *E. coli* [53].

In summary, this study provides a minimalistic, effective genetic engineering strategy for HA production in *B. subtilis*. By co-expressing hyaluronan synthase gene and a high-performance *tuaD* homolog, the study helped decrease precursor bottlenecks under the tested conditions, achieving shake-flask titers that are competitive with early non-pathogenic systems. The GRAS status, high secretory capacity and well-characterized genetics of *B. subtilis* suggest that this platform may represent a safer alternative to conventional animal-derived or pathogenic fermentation systems. The findings are similar to the authors' recent complementary studies on HA production systems. In those studies, other HA-producing strains were assessed under various conditions and the regulatory mechanisms of HA biosynthesis were investigated using genome-scale approaches [54, 55]. Insights from those studies contributed to the design and development of the engineered strain in the present study.

Although the present study was carried out at the shake-flask scale, scaling up HA production to bioreactor systems may introduce several engineering and physiological challenges. Accumulation of HA can increase broth viscosity; thereby, limiting mixing efficiency and oxygen transfer. The HA biosynthesis competes with cellular metabolism for key precursors such as UDP-GlcNAc and UDP-GlcUA, potentially imposing metabolic burden during high-density cultivation. Furthermore, agitation and aeration affect HA production and molecular weight, while excessive shear stress may damage cells and decrease polymer size. Accumulation of byproducts such as lactic acid may inhibit cell growth and HA synthesis, highlighting the need of careful bioprocess design during scale-up [19]. The high viscosity of HA-rich fermentation broths poses significant challenges for downstream processing, including filtration and recovery of HA. These challenges necessitate careful optimization of the purification steps, especially during scale-up to industrial production [56].

4. Conclusion

In this study, the dual-gene-engineered strain demonstrated enhanced HA yield, compared to the single-gene-engineered strain, highlighting the critical role of precursor supply in HA biosynthesis. Characterization of

the purified HA by FTIR verified its chemical identity, showing all expected functional groups, while GPC analysis revealed a predominantly HMW polymer fraction, suitable for food, biomedical and cosmetic uses. The findings establish this engineered *B. subtilis* platform for HA biosynthesis, with the potential to meet growing market demand for safe and high quality HA. This study highlights *B. subtilis* as a promising chassis for the safe and efficient biomanufacturing of high-value biopolymers.

In addition to the scientific and technical contributions, this study includes broader industrial significance. The establishment of a GRAS, non-pathogenic and secretion-competent *B. subtilis* platform provides a further safe, regulatory-friendly cost-effective alternative to animal-derived or pathogenic fermentation routes currently used in the biopharmaceutical and cosmetic industries. The ability to produce HMW-HA, a premium grade material for dermal fillers, wound healing products, moisturizers and functional foods, highlights the translational potential of this platform as a promising foundation for further industrial bioprocess development. However, translating this proof-of-concept into an industrially relevant platform needs further optimization of process scale-up, molecular weight control and plasmid stability.

Based on the limitations identified in this study, further research should focus on improving HA production in *B. subtilis* through metabolic engineering and process optimization. Although a titer of 1.20 g l⁻¹ was achieved at shake-flask scale without optimization, additional studies are needed to enhance production efficiency and assess plasmid stability during scale-up. Further studies investigate hydrolase gene deletion to improve molecular weight, optimization of fed-batch fermentation and engineering strategies to decouple production from sporulation for further scalable and stable processes.

5. Declaration

5.1. Acknowledgements

This study was carried out as a part of the first author's PhD project.

5.2. Declaration of competing interest

The authors declare no conflict of interest.

5.3. Authors' Contributions

Rouzbeh Almasi Ghale: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review & editing.

Reza Faghihi: Investigation; formal analysis; writing – original draft; visualization.



Marjan Talebi: Formal analysis; writing – original draft; writing – review & editing; visualization.

Mehdi Shamsara: Conceptualization; formal analysis; investigation; methodology; supervision; validation; writing – original draft; writing – review & editing.

Fatemeh Tabandeh: Conceptualization; formal analysis; investigation; methodology; project administration; funding acquisition; supervision; validation; writing – original draft; writing – review & editing.

5.4. Using Artificial Intelligent Chatbots

The authors used ChatGPT (OpenAI) for text refinement and improving readability of the manuscript. All scientific content, data interpretation and final responsibility for the manuscript are linked to the authors.

5.5. Ethical Consideration

None.

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سویه مهندسی شده و زیست‌ایمن از *باسیلوس سابتیلیس* ۱۶۸ برای زیست‌سنتز اسید هیالورونیک

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چکیده

سابقه و هدف: اسید هیالورونیک یک پلیمر با ارزش بالا است که به طور گسترده در صنایع غذایی، آرایشی-بهداشتی، درمانی و دارویی کاربرد دارد. هدف این مطالعه ایجاد یک سامانه زیست‌تولیدی ایمن برای تولید اسید هیالورونیک از طریق مهندسی *باسیلوس سابتیلیس* است.

مواد و روش‌ها: ژن سنتاز هیالورونان از *Streptococcus dysgalactiae* و هم‌تای ژنی *tuaD* با نام NX02_04625 از *Sphingomonas sanxanigenens* بر اساس کارایی کاتالیتیکی گزارش شده انتخاب شدند و توالی آن‌ها برای بیان در *باسیلوس سابتیلیس* بهینه‌سازی کدونی شد. سازه کامل ناقل pHT01 که حاوی هر دو ژن بود، به صورت تجاری سنتز شد و توالی آن تأیید گردید. همچنین یک پلاسمید تک‌ژنی ساخته شد و هر دو پلاسمید از طریق روش الکتروپوریشن به *باسیلوس سابتیلیس* منتقل شدند. مقدار تولید اسید هیالورونیک با آزمون CTAB اندازه‌گیری شد، ساختار آن با FTIR تأیید و ویژگی‌های وزن مولکولی آن با کروماتوگرافی اندازه‌ای بررسی شد.

یافته‌ها و نتیجه‌گیری: سویه حامل سازه دوزنی مقدار $1/20 \pm 0/03$ گرم بر لیتر اسید هیالورونیک تولید کرد که نسبت به سویه تک‌ژنی افزایش ۲/۴ برابری نشان داد. بررسی وزن مولکولی الگوی سه‌قله‌ای را نشان داد که عمدتاً شامل پلیمرهای با وزن مولکولی بالا ($1/18 <$ میلیون دالتون؛ $43/45\%$) بود و در کنار آن گونه‌های با وزن مولکولی متوسط (حدود $0/99$ مگادالتون؛ $24/69\%$) و الیگومرهای با وزن مولکولی پایین (حدود 55 کیلودالتون؛ $31/86\%$) مشاهده شد. به طور کلی، این یافته‌ها کارایی و ایمنی زیستی سامانه تولید مهندسی شده را نشان می‌دهد و با توجه به غالب بودن اسید هیالورونیک با وزن مولکولی بالا و ویژگی‌های ژل‌مانند مطلوب، پتانسیل آن را برای کاربردهای غذایی برجسته می‌سازند.

واژگان کلیدی: *باسیلوس سابتیلیس* ۱۶۸، ژن سنتاز هیالورونان، تولید اسید هیالورونیک، مهندسی متابولیک، توسعه سویه، UDP-گلوکز دهیدروژناز