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Production of High Molecular Weight Hyaluronic Acid via Recombinant *Bacillus subtilis* 1A752

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Abstract

This study aimed to synthesize hyaluronic acid (HA) using a non-pathogenic recombinant bacterial host, *Bacillus subtilis* 1A752. The HA synthase (*hasA*) gene, originally isolated from *Streptococcus equi subsp. zooepidemicus,* was introduced into *B. subtilis* 1A752. This host strain naturally contains functional analogs of the *Streptococcus* genes *hasB, hasC,* and *hasD*, namely *tuaD, gtaB,* and *gcaD*. The *hasA* gene (or operon) was amplified from *S. zooepidemicus* genomic DNA using polymerase chain reaction (PCR) and subsequently cloned into *B. subtilis* 1A752 through recombinant DNA technology. The engineered strain was then employed for HA production, followed by deproteinization of the product using the Sevag method and quantification via the Carbazole assay. The purified HA was characterized further through chromatographic techniques, with its molecular structure verified using Nuclear Magnetic Resonance (NMR) and Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy. The viscosimetric analysis determined the intrinsic viscosity (ηi) and molecular weight, ranging from 1.7 to 2.7 MDa. The synthesized HA exhibits promising potential for pharmaceutical, biomedical, and cosmetic applications with its high molecular weight and non-pathogenic production platform.

Keywords: Hyaluronic acid; Synthase; Recombinant *Bacillus subtilis* 1A752; Recombinant DNA technology; Recombinant HA production and characterization.

Introduction

HA is a natural biopolymer in the glycosaminoglycan (GAG) family. It is composed of repeating disaccharide units of glucuronic acid and N-acetyl-glucosamine, which are alternately linked by β (1→3) and β (1→4) glycosidic bonds. HA predominantly occurs as sodium hyaluronate; unlike other GAGs, it is never sulfated [1]. *In vivo*, the molecular weight of HA can vary significantly from 10^5 to 10^7 kDa with chain lengths ranging between approximately 2,000 and 50,000 disaccharide units. Due to its polyelectrolyte nature, HA expands into macromolecular coils in low-concentration aqueous solutions as a result of electrostatic repulsion among its carboxylate groups, leading to a marked increase in solution viscosity [2, 3]. HA's unique rheological and structural properties render it pivotal to the human body. HA contributes to tissue hydration and structural integrity and imparts essential mechanical properties such as shock absorption in cartilage and elasticity in blood vessels. Compared to collagen networks, HA's supramolecular organization helps define tissues' overall architecture. It

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is abundantly present in various tissues-including epithelial, connective, and nervous tissues and is highly concentrated in the vitreous humor of the eye, synovial fluid, and skin [4, 5]. Moreover, HA exists in different forms circulating freely, embedded in tissues, or as part of the extracellular matrix thereby continuously supporting structural preservation [6, 7]. Notably, over 50% of the body's HA is found in the skin, although its concentration declines with age, contributing to wrinkle formation [8].

Owing to its distinctive physico-chemical properties, HA has found extensive applications in medicine and cosmetics. This polysaccharide treats osteoarthritis, ophthalmic and plastic surgeries, wound healing, drug delivery, and skin hydration [9, 10]. Furthermore, the specific applications of HA are primarily determined by its molecular weight: higher molecular weight HA (>2,000 kDa) is favored in pharmaceutical and medical applications, whereas HA of lower molecular weight (<1,000 kDa) is generally preferred in cosmetic formulations [11]. For nearly three decades, the demand for HA in therapeutic applications has spurred the evolution of production methods. Initially, HA was predominantly sourced from animal tissues such as rooster combs, umbilical cords, and bovine vitreous humor [12]. However, issues including batch-to-batch variability, potential allergenicity, and ethical concerns have prompted a shift toward microbial production. Although enzymatic production is possible, its complexity and cost have limited widespread adoption. Bacterial production of HA has been known for over 50 years, with *Streptococcus* species (particularly those belonging to serological groups A and C) traditionally being the most common producers. Nevertheless, the use of *Streptococcus* strains carries a significant risk of bacterial toxin contamination [13-15]. Recombinant production systems employing non-pathogenic hosts, such as *B. subtilis*, have emerged as promising alternatives. *Bacillus* species are widely utilized in industry for producing enzymes, amino acids, vitamins, and other biochemicals due in part to their Generally Recognized as Safe (GRAS) status and ease of genetic manipulation [16].

Accordingly, the primary objective of this research was to develop recombinant strains of *B. subtilis* to produce high-quality HA. Specifically, the *hasA* operon responsible for HA synthesis was amplified from the genomic DNA of S. *equi subsp. zooepidemicus* and cloned into *B. subtilis* 1A752 using recombinant DNA technology. The engineered strain successfully produced HA under optimized culture conditions, yielding a product with a high molecular weight suitable for both pharmaceutical and cosmetic applications.

Material and Methods

Bacterial strains and Plasmids

Bacterial strain *B. subtilis* 1A752, used for HA production, was purchased from the Bacillus Genetic Stock Center (Columbus, Ohio, USA). The natural HA-producing strain *S. equi subsp. zooepidemicus* ATCC 15414 and *Escherichia coli* ECE 103, utilized for plasmid extraction, were obtained from the Ege University, Faculty of Science, Department of Biotechnology (Izmir, Türkiye). *E. coli* TOP10 (Invitrogen), employed for cloning, was obtained from Anadolu University, Faculty of Arts and Sciences, Department of Biology (Eskişehir, Türkiye).

Gene cloning by PCR

Genomic DNA was extracted from *S. equi subsp. zooepidemicus* ATCC 15414 using a genomic DNA purification kit (ThermoFisher, USA). PCR amplification was performed in a 25 µL reaction volume containing 1 ng of template genomic DNA, 2.5 µL of specific primers (Invitrogen, USA), 2 µL of dNTP mix (Invitrogen, USA), 0.2 µL of Taq polymerase, and 2.5 µL of Dream Taq polymerase (ThermoFisher, USA), with distilled water making up the final volume. PCR was carried out for 32 cycles with the following conditions: denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 10 seconds, using the primer pairs *rHasA*-F (*Bam*HI) and *HasA*-R (*Sph*I). The primer sequences were as follows:

The resulting 1250 bp PCR product was purified and digested with *Bam*HI and *Sph*I (ThermoFisher, USA).

***rHasA*-F (*Bam*HI):** CCGGGATCCAAAGGAGGTGATAAAAATGAGAACTAAAACCTCATAACT
***HasA*-R (*Sph*I):** CCGGCATGCTTAGTGGTGGTGGTGTAATTTTACGTGTTCCAGTC

Simultaneously, plasmid pMLK-83 was extracted from *E. coli* ECE 103 using the GeneJET Plasmid Miniprep Kit (ThermoFisher, USA). This plasmid, which contains a strong promoter (*amyE*) and multiple cloning sites (*Sal*I, *Cla*I, *Hind*III, *EcoR*I, *Pst*I, *Sma*l, *Bam*HI, *Sph*I, *Xba*I, *Not*I, *Xba*I, and *Eco*RI), has a total size of 9,900 bp (Table 1). The purified PCR product and the pMLK-83 plasmid were digested with *Bam*HI and *Sph*I. The digested product was then isolated from a 1% agarose gel using the GeneJET Gel Extraction Kit (ThermoFisher, USA) and ligated into the digested pMLK-83 vector using T4 DNA ligase (BioLabs, USA) at 16°C for 16 hours.

Using a standard transformation procedure, the recombinant plasmid, designated pMLK83-*has*A, was transformed into competent *E. coli* Top10 cells (Invitrogen). Transformants were selected on ampicillin-containing plates (10 µg mL^{-1}, Sigma-Aldrich, USA). Colony PCR was used to confirm the presence of the *has*A gene in positive clones. Finally, the verified pMLK83-*has*A plasmid was extracted from *E. coli* ECE 103, purified, and used for subsequent transformation into *B. subtilis* 1A752. Transformed *B. subtilis* strains were stored in saline containing 50% glycerol at -80°C for future use.

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HA production

All chemicals used for HA production were sourced from Sigma-Aldrich (USA) and Merck (Germany). Four different culture media were evaluated for their efficacy in HA production:

- **Luria Bertani (LB) Medium** (g L^{-1}): tryptone, 10; yeast extract, 5; and NaCl, 10.
- **Basal Medium (BM)** (g L^{-1}): yeast extract, 5; malt extract, 3; peptone, 5; KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 1; and vitamin B1 hydrochloride, 0.01 (added post-autoclaving).
- **Modified Minimal Broth (MMB)** (g L^{-1}): tryptone, 2; K_2HPO_4, 7; KH_2PO_4, 2; $Na_3C_6H_5O_7$, 0.5; $(NH_4)_2SO_4$, 2; and $MgSO_4$, 0.5.
- **Tryptic Soy Broth (TSB)** (g L^{-1}): Pepton from casein, 17; pepton from soymeal, 3; D(+)-Glucose monohydrate, 2.5; sodium chloride (NaCl), 5; di-potassium hydrogen phosphate, 2.5.

The initial pH of each culture medium was adjusted to 7.0 before sterilization at 121°C for 20 minutes. Glucose was incorporated into all media as the carbon source at a concentration of 1% (w/v).

Recombinant *B. subtilis* 1A752 was cultured in an orbital shaker (New Brunswick™ Scientific Excella E24, Eppendorf, Germany) at 37°C, initially at a shaking speed of 150 g. The culture was started with 25 mL of LB medium, and 2 mL of this culture was inoculated into each of the four media for HA production. The incubation period was extended to 48 hours at 37°C, while the shaking speed was increased to 175 g during the production phase.

HA extraction and quantification method

HA produced by the mutant *B. subtilis* strain 1A752 in broth cultures was quantified using the Carbazole method, initially described by Bitter and Muir (1962) [17]. This widely cited method has been modified slightly to improve HA recovery during purification.

The production medium (50 mL) was first sterilized by filtration through a 0.45 µm filter and then precipitated using cetylpyridinium chloride (CPC) at 1 mg mL^{-1} concentration. The mixture was incubated at 37°C for 12 hours to allow precipitation. The precipitate was redissolved in 0.5 M NaCl, after which two volumes of ethanol were added. The resulting mixture was left to stand at 4°C for 12 hours. Subsequently, the precipitate was collected by centrifugation at 6,000 g for 20 minutes at room temperature and resuspended in NaCl solution. Following four to five ethanol washes, the final HA pellet was diluted with 4 mL of sterile water for subsequent HA analysis.

A standard curve for glucuronic acid was generated using commercial glucuronolactone (Sigma-Aldrich, USA) at concentrations ranging from 4 to 40 µg mL^{-1}. In a glass tube chilled to 4°C, sodium tetraborate was added. The assay sample or standard was added; the tube was then sealed, shaken, and incubated for 10 minutes at 100°C. After cooling to room temperature, Carbazole (0.125% in ethanol) was added, and the tubes were shaken and incubated at 100°C for an additional 15 minutes. Absorbance was measured at 530 nm using a PerkinElmer Lambda™ 35 UV/VIS spectrophotometer.

The assay's limitation lies in its non-specificity, as other compounds (e.g., glucose) may also react. Therefore, a background reading was determined post-HA isolation by precipitation. The HA titer was calculated to be 2.05 times the glucuronic acid titer, as reported by Boeriu et al., Widner et al., and Yu and Stephanopoulos [13, 15, 18]. All quantification assays were performed in triplicate, and the data were averaged.

Finally, samples were concentrated and re-precipitated with 1 mg mL^{-1} CPC to purify the HA further. The resulting precipitate was redissolved in 0.5 M NaCl, mixed with two volumes of ethanol, and incubated at 4°C for 12 hours. After centrifugation, the final pellet was resuspended in deionized water for further analysis.

Deproteinization and purification of HA

A 10 mL aliquot of aqueous HA was deproteinized using the Sevag method by adding three volumes of a chloroform/n-butanol mixture prepared in a 4:1 ratio [19]. After vigorous vortexing for 5 minutes at room temperature, the mixture was allowed to stand for 15 minutes to facilitate phase separation. The organic phase, which contained the residual proteins, was discarded. This deproteinization step was repeated three times.

Protein contamination in the HA samples was quantified using the Bradford method [20]. To the remaining aqueous phase, two volumes of ethanol were added and the mixture was thoroughly mixed before storage at 4°C for 12 hours. The mixture was centrifuged at 6,000 g for 15 minutes at room temperature, and the supernatant was discarded. The resulting HA pellet was then subjected to further purification.

For the first purification phase, ion exchange chromatography (IEC) was employed using a DEAE-Sepharose CL-4B column (1.5 cm x 30.0 cm; ISOLAB, Germany). Elution was performed at room temperature using a linear gradient of NaCl solutions at concentrations of 0, 0.25, 0.50, 0.75, 1.00, and 1.50 M, with a flow rate of 0.8 mL/min. One-milliliter fractions were collected, and their absorbance was measured at 530 nm. Fractions exhibiting HA activity were pooled, re-precipitated with ethanol, and resuspended in deionized water. Further purification of these fractions was achieved through dialysis using a Spectra/Por™ membrane.

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For the second purification phase, gel filtration chromatography (GFC) was performed on a Sephadex G-75 column (Sigma-Aldrich, USA) using the exact column dimensions. Elution was carried out at a 0.6 mL/min flow rate with a 0.75 M NaCl solution as the eluent. Again, 1 mL fractions were collected, and their absorbance was measured at 530 nm. The active HA fractions were combined, re-precipitated with ethanol, resuspended in deionized water, and subjected to dialysis.

Finally, the purified HA was lyophilized using a MicroModulyo freeze-dryer (Thermo Electron Corporation, USA) at -45°C and 50 mBar.

HA characterization

A viscometrical method was employed to determine the molecular weight of the produced HA. In addition, NMR and FTIR spectroscopy were used to characterize the chemical structure, identify functional groups, and assess the degree of purity.

Molecular weight determination

The molecular weight of HA was determined using viscometry, following the method described by Mei et al. (2019) [10]. HA solutions at concentrations of 0.05%, 0.10%, 0.15%, and 0.20% (w/v) were prepared in 0.10 M NaCl. Viscosity measurements were performed at 25°C using an ALPHA SERIES rotary viscometer (Fungilab, Spain) equipped with a model bar SP:L1 at a rotational speed of 100 rpm. Each measurement was repeated three times to obtain the mean viscosity (η).

The relative viscosity (ηr) was calculated using the equation:

$$\eta r = \eta[HA] / \eta[NaCl] \quad (1)$$

The specific viscosity (ηsp) was then computed by subtracting one from ηr and dividing by the HA concentration (expressed as a fraction), as follows:

$$\eta sp = (\eta r - 1) / (\%HA) \quad (2)$$

Standard plots of both ηr and ηsp versus HA concentration were generated. The ηi was determined by extrapolating the plot of ηsp as a function of HA concentration to zero concentration (i.e., the y-intercept of the plot).

Finally, the molecular weight (Mw) of HA was calculated using the Mark-Houwink-Sakurada equation [21]:

$$\log(\eta i) = \log K + \alpha \log(Mw) \quad (3)$$

For Laminaria-type HA, the following parameters were applied:

- For low molecular weight HA: $K = 3.39 \times 10^{-4}$ and $\alpha = 0.79$
- For high molecular weight HA: $K = 3.95 \times 10^{-3}$ and $\alpha = 0.60$

^{1}H NMR spectroscopy of HA

^{1}H NMR spectroscopy was performed using a JNM-ECZS 400 MHz NMR spectrometer (Jeol, USA). Sodium trimethylsilyl propionate served as the internal reference, and deionized water was used as the solvent for the HA sample. The analysis was conducted at a HA concentration of 1 mg mL^{-1}.

ATR-FTIR spectroscopy of HA

ATR-FTIR spectroscopy was employed to measure the vibrational frequencies of HA bonds, facilitating the identification of relevant functional groups. The analysis was conducted using a DTGS FTIR spectrometer with a spectral range of 600–4000 cm^{-1} (PerkinElmer, UK) and utilized 2 mg of HA for the measurement.

Results and Discussion

Gene cloning by PCR and bacterial transformation

In this study, an artificial operon containing the *hasA* gene (Figure 1) was engineered and incorporated into the integration plasmid pMLK-83. The *hasA* gene, isolated from *Streptococcus equisimilis*, has been demonstrated in previous studies to produce high-quality HA when expressed in *B. subtilis* strains [14-16, 22]. The native *S. equisimilis* operon exhibits a high intrinsic polymerization rate. It comprises four genes: *hasA* (coding for HA synthase), *hasB* (coding for UDP-glucose dehydrogenase), *hasC* (coding for UDP-glucose pyrophosphorylase), and *hasD* (coding for UDP-N-acetylglucosamine pyrophosphorylase). In *B. subtilis*, homologous genes are designated as *tuaD, gtaB*, and *gcaD* [15, 16]. These genes also involve critical cellular functions such as cell wall synthesis, glycolysis, and the pentose phosphate pathway.

The selected *B. subtilis* 1A752 strain was chosen for its advantageous growth characteristics and high yield of secreted proteins in industrial fermenters. The integration plasmid pMLK-83, which features a strong promoter (Table 1), was isolated from *E. coli* ECE 103 and used to drive HA biosynthesis. Subsequently, the engineered pMLK-83-*hasA* construct was introduced into *E. coli* Top10 cells using standard transformation methods [23]. Transformation efficiency was assessed on agar plates containing ampicillin, and representative colonies were randomly selected. PCR amplification of these colonies confirmed the successful integration of the *hasA* gene. The recombinant pMLK-83-*hasA* plasmid was extracted from *E. coli* Top10, purified, and subsequently transformed into *B. subtilis* 1A752 for HA production. Successful HA synthesis in the transformed *B. subtilis* strain confirmed the functional expression of the *HasA*

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enzyme, paving the way for further in-depth characterization of the synthesized HA.

Table 1: Characteristics of pMLK-83 plasmid

amyE	*amy 5'*and *3'* parts of *B. subtilis* α amylase gene
neo	Encode; selectable in either *E. coli* or *B. subtilis* (neomycin or kanamycin 5μg ml^{-1})
bla	Encode β-lactamase; selectable in *E. coli* only (ampicillin 50μg ml^{-1})
gusA	Encode β-glucuronidase reporter

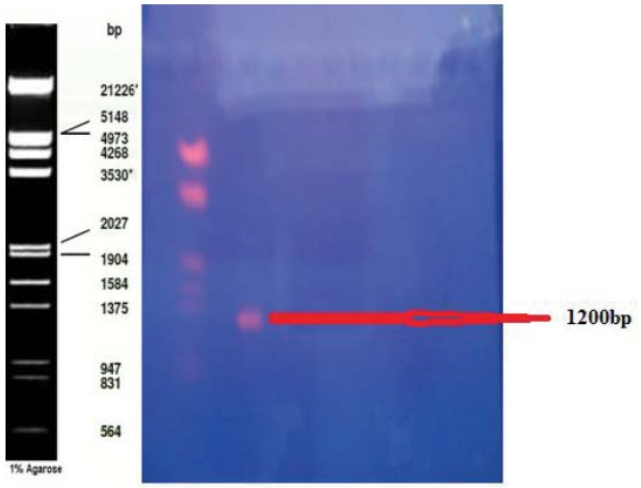

Figure 1: shows the *has*A gene analyzed on a 1% agarose gel at 100 V. Fermentas Lambda DNA/*Eco*RI+*Hind*III, which was used as a marker. The gel image shows that the *has*A gene measures approximately 1,200 bp.

B. subtilis has been employed in protein production for many years due to its ease of cultivation, adaptability to genetic modifications, and its status as a non-pathogenic organism. Today, enzymes produced by *Bacillus* species are extensively used in the production of cheese, wine, bread, and other consumable products, as well as in both agricultural and biomedical applications.

HA production

Numerous studies have examined the influence of culture medium composition and various physico-chemical parameters, such as agitation speed, oxygenation, pH, and temperature, on HA production [24, 25]. To optimize microbial growth and HA production conditions, four different culture media-LB, BM, MMB, and TSB-were evaluated. Glucose, at a concentration of 1%, served as the carbon source in the culture medium, and incubation took place for 48 hours at 175 *g*. Figure 1 presents the results obtained from these four-culture media. According to the results, the highest HA yield was achieved using BM medium, with a concentration of 195.45 mg L^{-1}.

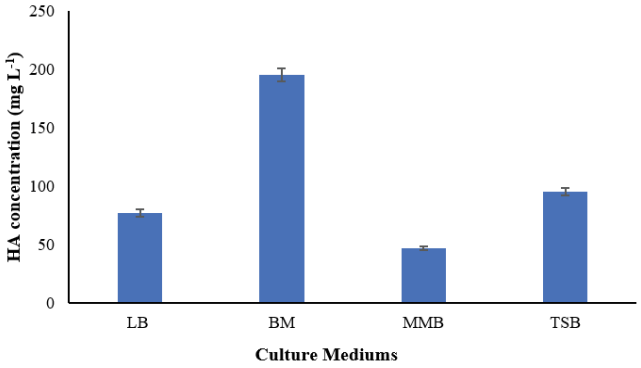

Figure 2: The HA titer produced by recombinant *B. subtilis* 1A752 strain in different culture media (37°C, 48 hrs, 170 g).

In contrast, HA production in LB, MMB, and TSB media yielded lower concentrations: 77.01 mg L^{-1}, 47.04 mg L^{-1}, and 95.10 mg L^{-1}, respectively. Based on these findings, the BM medium was identified as the optimal culture medium for growth and HA production in the recombinant *B. subtilis* 1A752 strain.

The BM medium not only produced the highest HA yield but also offers advantages in terms of simplicity and cost-effectiveness. Previous studies have often employed complex media rich in nitrogen, carbon, amino acids, minerals, and vitamins for HA production. For instance, Chong and Nielsen used 50 g L^{-1} maltose as a carbon source and 20 g L^{-1} yeast extract as a nitrogen source in *S. equi subsp. zooepidemicus* (ATCC 35246) [26]. Liu et al. utilized 70 g L^{-1} sucrose and 25 g L^{-1} yeast extract with *S. equi subsp. zooepidemicus* WSH-24 [27]. Similarly, Pan et al. (2015) used 30 g L^{-1} glucose and 30 g L^{-1} yeast extract with *S. equi subsp. zooepidemicus* (ATCC 39920) [28].

Contrary to previous studies, in this research, we utilized only 10 g L^{-1} of glucose and a minimal quantity of essential elements for bacterial growth to yield a significant amount of HA. Additionally, the microorganism we employed for this production is recognized as non-pathogenic, in contrast to the pathogenic *Streptococcus* species, which are natural HA producers. *Streptococcus* can produce vast amounts of endotoxins and exotoxins, substances detrimental to human health. Considering these findings, recombinant strains of *B. subtilis* have the potential to produce HA in large quantities, at high quality, and more economically.

HA extraction and quantification method

Following centrifugation at 9,000 *g* for 20 minutes, the microorganisms were separated from the culture medium, which was then sterilized using filtration. HA was precipitated by adding CPC, followed by the addition of two volumes of ethanol. This mixture was then incubated at 4°C for 12 hours. The pellet was collected by centrifuging at 6,000 *g* for 15

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minutes, resuspended in NaCl, and subsequently washed with ethanol. For HA analysis, the pellet was diluted with sterile water. The HA concentration was determined using the method developed by Bitter and Muir (1962) [17]. In this assay, the β-(1,4)-D-glucuronic acid units present in the HA structure react with Carbazole, resulting in a purple color change as a qualitative indicator (Figure 3). Spectrophotometric measurements were taken at an absorbance of 530 nm to ascertain the glucuronic acid concentration (Figure 4). This concentration was then compared to a glucuronolactone standard curve, prepared in a range from 4 to 40 µg mL^{-1}, to calculate the exact amount of glucuronic acid present.

The HA was quantified in triplicate, and the data were averaged across all tests. According to the protocol, the HA titer is 2.05 times the measured glucuronic acid titer [13, 15, 18].

Figure 3: Qualitative determination of HA.

Deproteinization and purification of HA

The deproteinization method Sevag developed applies to all polysaccharides [29, 30]. A mixture of Sevag reagents ($CHCl_3$/n-BuOH, 4:1 ratio) was added to the aqueous HA solution and vigorously stirred for 5 minutes. After allowing the mixture to stand for 15 minutes, the two phases separated: protein residues migrated into the organic phase,

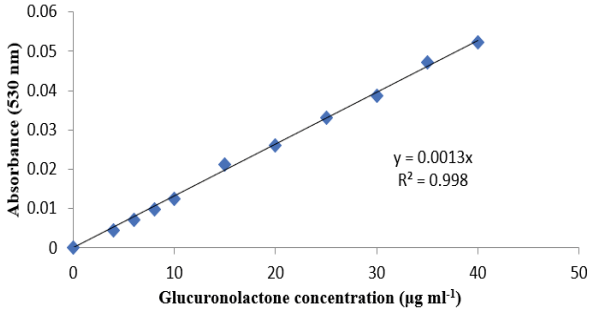

Figure 4: Glucuronolactone standard (4-40 µg ml^{-1}).

while the polysaccharides remained in the upper, aqueous phase. Following removing the organic phase, two volumes of ethanol were added to the remaining aqueous phase. The mixture was then stored in a refrigerator at 4°C for 2 hours. The residual HA in the aqueous phase was recovered by centrifuging at 6,000 *g* for 15 minutes. The supernatant containing ethanol was removed, and 5 mL of deionized water was added to the HA pellet for resuspension.

Subsequent purification procedures were performed, first by conducting semi-purification using IEC, followed by GFC for further refinement.

Purification of HA by IEC on DEAE-Sepharose CL-4B

Four mL of the HA solution was loaded onto a DEAE-Sepharose CL-4B column prepared for ion exchange chromatography. NaCl solutions ranging in concentration from 0 to 2.00 M (i.e., 0, 0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 M), prepared in deionized water, served as eluents. Elution was performed using a linear gradient at a flow rate of 0.80 mL/min. A total of 60 fractions were collected, and the absorbance of each fraction was measured at 530 nm; the resulting data was then plotted on a graph.

The highest absorbance peaks, observed in fractions 26 to 56, indicated the presence of HA. Notably, these peaks were detected when using a 0.75 M NaCl solution, corresponding to the point of maximum ion saturation (Figure 5). Fractions exhibiting HA activity were collected, re-precipitated using CPC and ethanol, and subsequently dialyzed. The resulting HA pellet was then dissolved in 4 mL of 0.1 M NaCl solution in preparation for GFC.

Purification of HA by GFC on Sephadex G75

After the IEC purification step, the HA extract was loaded onto a Sephadex G-75 column prepared for GFC. A 4 mL aliquot of the HA sample was applied to the column, and a 0.75 M NaCl solution (prepared in deionized water and previously determined to provide maximum ion saturation during IEC) was used as the eluent. Elution was performed at a flow rate of 0.60 mL/min. A total of 45 fractions were

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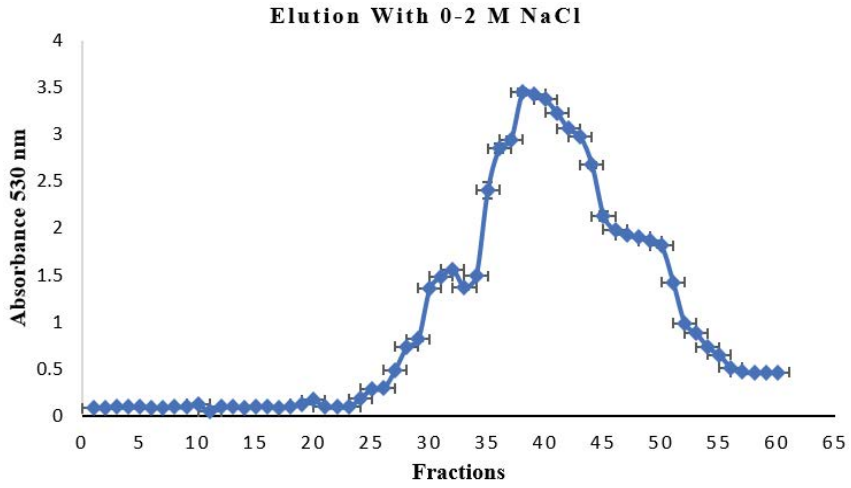

Figure 5: Fractionation model by IEC on DEAE-Sepharose CL-4B (1.5 cm x 30.0 cm) of HA produced by *B. subtilis* 1A752. The column was washed with pure water. 0-2 M NaCl concentrations were used as eluent. The maximum saturation was obtained with a concentration of 0.75 M NaCl.

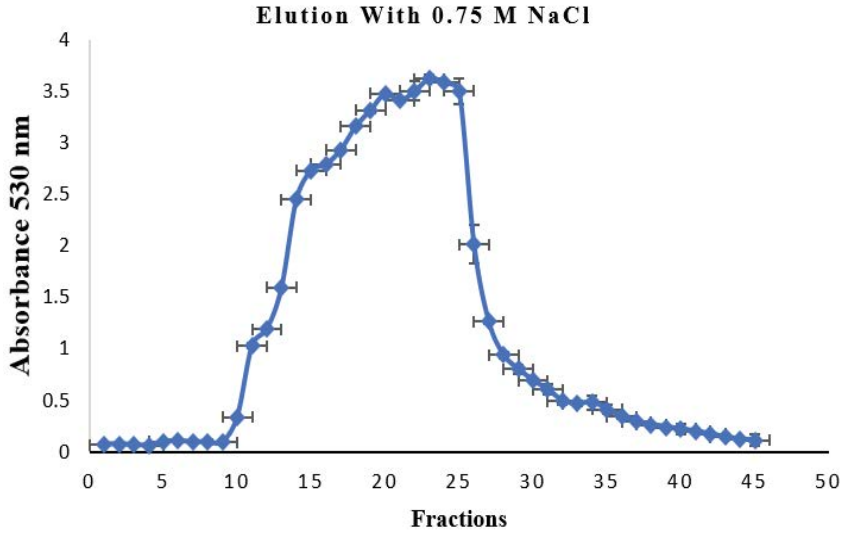

Figure 6: Fractionation model by GFC on Sephadex G75 (1.5 cm x 30.0 cm) of HA produced by *B. subtilis* 1A752. The column was washed with pure water. 0.75 M NaCl served as the eluent.

collected, and the absorbance of each fraction was measured at 530 nm; the data was then plotted on a graph (Figure 6). The highest absorbance peaks, observed between fractions 10 and 36, indicated the presence of HA. Fractions exhibiting HA activity were collected and precipitated using CPC and ethanol, and the resulting HA pellet was dissolved in deionized water. Finally, the purified HA was lyophilized for further analysis.

HA characterization

Molecular weight determination

Tables 2 and 3 present the viscosity data for NaCl and HA solutions. The viscosity measurements were conducted using a Fungilab ALPHA series rotational viscometer (model SP: L1). To calculate the relative viscosities (ηr), the mean viscosity values for HA were divided by the mean viscosity of a 0.10 M NaCl solution. The specific viscosities (ηsp) were subsequently calculated by subtracting one (1) from the ηr and then dividing by the HA concentration (Table 3).

The ηi was determined by plotting the norms of the ηsp against their corresponding concentration values, as shown in Figure 7. By extending the line of best fit to the y-axis, the value of ηi was ascertained. This ηi value was then used in the Mark-Houwink-Sakurada equation to calculate the molecular weights of HA.

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Table 2: Viscosity Values of HA Solutions in Different Concentrations

Viscosity	0.10M NaCl	0.05% HA	0.10% HA	0.15% HA	0.20% HA
1. Trial	8.06	19.67	30.84	41.27	46.52
2. Trial	8.08	19.88	30.72	41.91	46.03
3. Trial	8.13	19.70	30.42	41.56	45.78
Average	**8.09**	**19.75**	**30.66**	**41.58**	**46.11**

Table 3: Relative and Specific Viscosity Values

% HA	$\eta r = \frac{\eta[HA]}{\eta[NaCl]}$	$\eta sp = \frac{\eta r - 1}{\%HA}$
0.05	2.44	28.80
0.10	3.79	27.90
0.15	5.01	26.73
0.20	5.70	28.50

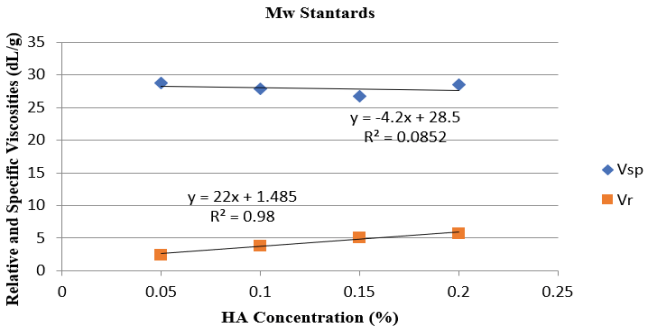

Figure 7: Standards of Relative and Specific Viscosities of HA in Different Concentrations.

While the R^2 value for the ηsp of HA is relatively low based on the obtained results, the R^2 value for the ηr is considerably high. However, the Mark-Houwink-Sakurada equations are only applicable to ηsp. Moreover, all ηsp values are derived by dividing the ηr values by the HA concentration percentage.

In the Mark-Houwink-Sakurada equation, the molecular mass is calculated using the ηi. It is characterized by two constants: K, a proportional constant dependent on solvent conditions, and α, the Staudinger index, which depends on polymer flexibility. For low-molecular-weight HA, α=0.79 with a constant K=3.39×10^{-4}. For high molecular weight HA, α=0.60 with a constant K=3.95×10^{-3}. These values suggest that low molecular weight HA is relatively rigid, while high molecular weight HA is more flexible.

The molecular weights for both low and high molecular weights were summed and then divided by two to obtain the average molecular weight. In this study, the ηi taken at the intersection was 28.50. It is worth noting that the intersection value for ηr (1.485) is not to be considered for these calculations.

Using the Mark-Houwink-Sakurada equation, the molecular weight of HA was calculated as follows:

Log [η_i] = Log K + αLog Mw (Mark-Houwink-Sakurada equation)

= 3.39 x 10^{-4} Mw$^{0.778}$ (10^5 < Mw < 10^6 Da)

= 3.95 x 10^{-3} Mw$^{0.604}$ (Mw > 10^6 Da)

K: Proportional constant (dL/g); α: Staudinger index; Mw: Molecular weight (Da); intrinsic viscosity [η_i] = 28.5 dL/g. This value is replaced by the Mark-Houwink-Sakurada equation.

In step 1: α = 0.79 and K = 3.39 * 10^{-4} values were applied to low Mw HA:

[η_i] = 28.5 α = 0.79 K= 3.39*10^{-4}

Log 28.5 = Log 3.39*10^{-4} + 0.79 Log Mw

Mw=$10^{6.23}$

Mw = 1,698,244 Da

In step 2: α = 0.6 and K = 3.95*10^{-3} values were applied to AH at high Mw:

[η_i] = 28.5 α = 0.6 K= 3.95*10^{-3}

Log 28.5 = Log 3.95*10^{-3} + 0.6 Log Mw

Mw=$10^{6.43}$

Mw = 2,691,535 Da

The molecular weights of HA produced in this study were 1.7 MDa and 2.7 MDa, with an average molecular weight of approximately 2.20 MDa. This was achieved by substituting the ηi value of 28.50 dL/g into the Mark-Houwink-Sakurada equation.

Compared to other studies on recombinant HA production, our results are promising. Widner et al. utilized recombinant *B. subtilis* 168 (*has*A-*has*B-*has*C-*has*D) and achieved molecular weights ranging from 1.1×10^6 to 1.2×10^6 Da [15]. Mao and Chen used recombinant *Agrobacterium sp.* (pm*Has*-kfiD) and observed molecular weights between 0.7×10^6 and 2.0×10^6 Da [31]. Yu and Stephanopoulos employed recombinant *E. coli* (*ssha*sA-*ssug*D) and reported molecular weights ranging from 3.5×10^5 to 1.9×10^6 Da [18]. Chen et al. used recombinant *S. equi subsp. zooepidemicus* (*has*A-*has*B-*has*C-*has*D -*has*E) to obtain molecular weights between 1.8×10^6 and 2.4×10^6 Da [24]. Our recombinant *B. subtilis* 1A752 (*has*A) study yielded HA with molecular weights ranging from 1.7×10^6 to 2.7×10^6 Da, demonstrating a significant improvement.

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Given these findings, recombinant strains of *B. subtilis* offer a promising avenue for HA production in large quantities and with high molecular weights. This could have considerable implications for medical, pharmaceutical, and cosmetic applications.

^{1}H NMR spectroscopy of HA

This study's ^{1}H NMR spectra of the HA produced displayed characteristic peaks, affirming its structural integrity. Specifically, a peak at 1.9 ppm was observed, which is attributed to the protons of the -CH_3 group. Signals between 3.1 and 5.6 ppm indicated D-glucuronic acid and N-acetyl glucosamine units. The signal at 4.7 ppm represented the solvent peak (dH_2O/D_2O) (Figure 8). These observations are in line with previously reported spectral data [32, 33]. NMR spectra were recorded at a 27–28°C temperature range using 5 mm Wilmad 528-PP NMR tubes. The data were analyzed using standard VARIAN software, and 2D spectra were acquired using correlated spectroscopy techniques in absolute value mode.

Our findings suggest that the HA produced by the recombinant *B. subtilis* 1A752 strain in shaking culture possesses an exceptionally high degree of purity. Notably, its structural characteristics are consistent with HA produced by *Streptococcus* strains, as reported in the literature [33-35]. This consistency is significant because the molecular structure of HA is highly conserved across evolutionary boundaries - from *Pseudomonas* to mammals, including rats and humans [36]. Such evolutionary conservation is believed to contribute to HA's favorable biocompatibility, low immunogenicity, and low risk of allergic reactions, making it particularly useful in medical, pharmaceutical, and cosmetic applications.

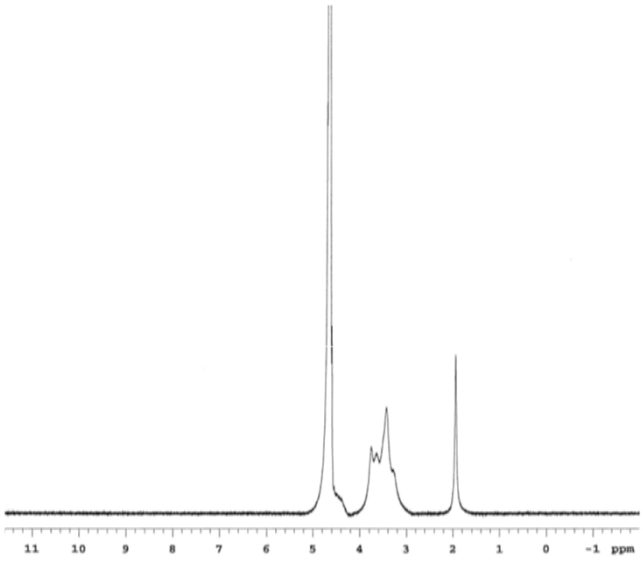

Figure 8: ^{1}H NMR spectroscopy of HA.

ATR-FTIR spectroscopy of HA

The ATR-FTIR spectrum of the HA produced in this study is shown in Figure 9. The spectrum reveals a peak at 3336 cm^{-1} associated with the intra- and intermolecular stretching vibrations of the −OH group. This corresponds to the stretching vibration of the hydrogen bonds in the −NH group. Another peak is observed at 2,878 cm^{-1}, which is attributed to the symmetrical stretching vibrations of the −CH2 group. Additionally, a peak at 1,557 cm^{-1} is indicative of the asymmetrical stretching of the CO in the COO^{-} group, which can accept one or two hydrogen bonds. Bands at 1,634 cm^{-1} and 1,481 cm^{-1} correspond to the symmetrical and asymmetrical vibrations of the COO^{-} carboxyl group, respectively (Figure 9). The peak at 1,064 cm^{-1} is associated with the saccharide units in the C−O−C hemiacetal system [32, 33]. Furthermore, the FT-IR spectrum overlaps the −CH stretch from the derivative structure with the −OH group of the HA. The C=C and C=O stretch also overlap with the C=O stretches in the HA structure.

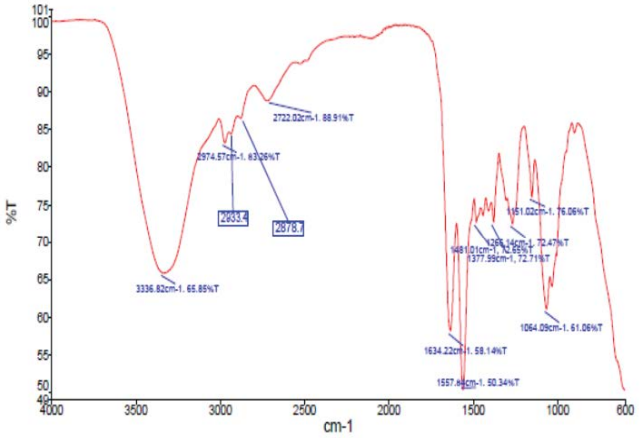

Figure 9: ATR-FTIR spectra of HA.

Conclusion

This study aimed to synthesize hyaluronic acid (HA) using a non-pathogenic recombinant bacterial host, *Bacillus subtilis* 1A752. The HA synthase (*has*A) gene, originally isolated from *Streptococcus equi subsp. zooepidemicus*, was introduced into *B. subtilis* 1A752. This host strain naturally contains functional analogs of the *Streptococcus* genes *has*B, *has*C, and *has*D, namely *tua*D, *gta*B, and *gca*D. The *has*A gene (or operon) was amplified from *S. zooepidemicus* genomic DNA using polymerase chain reaction (PCR) and subsequently cloned into *B. subtilis* 1A752 through recombinant DNA technology. The engineered strain was then employed for HA production, followed by deproteinization of the product using the Sevag method and quantification via the Carbazole assay. The purified HA was characterized further

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through chromatographic techniques, with its molecular structure verified using Nuclear Magnetic Resonance (NMR) and Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy. The viscosimetric analysis determined the intrinsic viscosity (ηi) and molecular weight, ranging from 1.7 to 2.7 MDa. The synthesized HA exhibits promising potential for pharmaceutical, biomedical, and cosmetic applications with its high molecular weight and non-pathogenic production platform.

It is important to note that hyaluronic acid (HA) was originally sourced primarily from animal tissues, such as rooster combs, umbilical cords, and bovine vitreous humor. However, the presence of allergies to animal products in some individuals has prompted researchers to explore bacterial production of HA for over 60 years. The microorganisms predominantly utilized for HA production are bacteria from the genus *Streptococcus*, which are generally pathogenic and can produce both exotoxins and endotoxins.

The primary risk associated with the microbial production technique is contamination by bacterial toxins. In the past 15 years, however, the production of HA using recombinant microorganisms has begun to replace the traditional methods involving *Streptococcus*. This shift helps avoid the risk of toxin contamination, reduces production and purification costs, and enhances the overall quality of the produced HA. Numerous studies have been conducted and published on this topic.

The originality of this current work lies in several key aspects:

- A bacterial strain with a notably high capacity for HA production was selected and subsequently sectioned by the Bacillus Genetic Stock Center (Columbus, Ohio, USA). This particular species of *Bacillu*s is utilized industrially to produce various products, including proteases, alpha-amylases, amino acids, and vitamins.

The *has*A operon, responsible for HA synthesis, was amplified from the genomic DNA of *S. zooepidemicus.* Utilizing recombinant DNA technology, this gene was successfully cloned into *B. subtilis* 1A752. The resulting recombinant strain of *B. subtilis* 1A752 produced HA effectively under optimized culture conditions.

Despite this study's straightforward extraction, purification, and quantification methods, we utilized a simple deproteinization and purification approach that could be easily adapted for industrial HA production.

In this work, we have shown that a simple and economical laboratory purification method, such as IEC on DEAE-Sepharose CL-4B (Sigma-Aldrich, USA) and GFC on Sephadex G-75 (Sigma-Aldrich, USA), was used.

- Although in most literature, NMR and FTIR spectroscopies have been used to characterize the chemical structure, identify functional groups, and obtain information on the degree of purity. In this work, we tried to adapt the viscometrical method while using mathematical formulas to determine the molecular weight. This method is a great novelty in the determination of the molecular weight of HA.

- In short, the production, purification, and characterization method of HA in this work was simple and easy, the production strain was very safe, and the HA produced in this work was of high quality. NMR and FTIR spectroscopies have sufficiently proven that the chemical structure and functional groups of the HA produced in this work were identical to the original. The viscometrical method used to determine the molecular weight of the HA product has sufficiently proven that the molecular weight was very high. All these factors show that the HA produced in this work was of high quality. The work showed that the *B. subtilis* 1A752 strain is a potential candidate to be adapted for producing higher-quality HA. This work paves the way for other research and sufficiently demonstrates that the HA produced by *the B. subtilis* 1A752 strain can be suitable for applications in pharmaceutical and cosmetic industries.

This text provides valuable and straightforward information on producing high molecular weight HA from *B. subtilis* 1A752.

Our findings indicate that the *B. subtilis* 1A752 strain can produce over 195 mg L^{-1} of HA. While molecular weight is typically determined through size-exclusion chromatography coupled with a refractive index detector, we utilized a viscometrical method. The NMR results revealed a high degree of purity. The molecular weight of the produced HA ranges from $1.7x10^6$ to $2.7x10^6$ Da. This contrasts with commonly used *Streptococcus* strains, which produce HA with average molecular weights ranging from $1.0x10^3$ to $4.3x10^6$ Da for natural strains and $3.5x10^5$ to $2.4x10^6$ Da for recombinant strains. High molecular weight HA poses a significant challenge, especially in the medical, pharmaceutical, and cosmetic sectors. Currently, various HA-based products are available in the market. This study proves that microbial production methods, particularly those using recombinant strains like *B. subtilis* 1A752, offer an affordable yet high-quality alternative for HA production.

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