Supplementary Materials

This supplementary file provides additional experimental details, raw data, and supporting figures related to the manuscript. These materials are provided to ensure transparency, reproducibility, and clarity of the experimental procedures and results described in the manuscript.

All supplementary content is organized to correspond directly with the sections cited in the main manuscript.

This file is intended to support the peer review process and facilitate future replication efforts, reflecting the authors' commitment to open and reproducible science.

Table S1. Reagents, kits, and Cloning Workflow

Item	Supplier	Catalog/Lot	Purpose/Notes
FavorPrep Gel/PCR Purification Mini Kit	Favorgen Biotech Corp. (Taiwan)	Cat. No: FAGCK001-2	Gel extraction of (backbone + *hasA*)
Plasmid DNA Extraction Mini Kit	Favorgen Biotech Corp. (Taiwan)	Cat. No: FAPDE050	Plasmid preparation
XbaI restriction enzyme	Thermo Fisher Scientific (USA)	10 U/µL; Tango Buffer Lot: 01258845	Excision of NX02_04625
T4 DNA Ligase	Thermo Fisher Scientific (USA)	Lot: 00024630; 200 µg/µL, 40,000 U	Ligation
10× T4 Ligase Buffer	Thermo Fisher Scientific (USA)	Lot: 00296988	Ligation buffer

Ampicillin; Chloramphenicol	—	—	Selection antibiotics for *E. coli* / *B. subtilis*
Gene synthesis and vector construction (dual-gene)	Gene Universal Inc. (USA)	Invoice SG11275; represented by Pars Biotek (Iran)	pHT01-*hasA*-NX02_04625
Primer design (colony PCR)	Gene Fanavaran Iranian Co.	—	Gene-specific primers for verifying single-gene (*hasA*) and dual-gene (*hasA* + NX02_04625) constructs

All reagents were molecular biology grade. Standard laboratory consumables (e.g., tubes, tips, agarose, electrophoresis buffers) were used according to manufacturer specifications.

Table S2. Gene Features, Enzymatic Parameters, and Vector Design

Gene	Organism/Accession	Enzyme/EC	Length (bp/aa)	MW (kDa)	Key parameters/design
hasA	*Streptococcus dysgalactiae* ATCC12394	Hyaluronan synthase; EC 2.4.1.212	1254 bp / 417 aa	~47.8	TN ≈ 120 (BRENDA); codon-optimized for *B. subtilis*
NX02_04625 (*tuaD* homolog)	*Sphingomonas sanxanigenens* DSM19645; UniProt W0A856	UDP-glucose 6-dehydrogenase; EC 1.1.1.22	1365 bp / 454 aa	~48	Low Km and high TN (BRENDA); start codon GTG→ATG to enhance translation

Additional vector features: pHT01 backbone; IPTG-inducible Pgrac01 promoter; intergenic RBS enabling independent translation; small expression tags included for protein differentiation; cloning restriction sites (e.g., BamHI, SmaI); final dual-gene construct length 10,782 bp.

Table S3. Media, Buffer Compositions, and Antibiotic MIC

Name	Composition
LB	Yeast extract 5 g/L, Peptone 10 g/L, NaCl 10 g/L
LBSP	Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 5 g/L, Sucrose 250 mM, Potassium phosphate buffer 50 mM (pH 7.2)
LBSPG	LBSP supplemented with 10% (v/v) glycerol
0.7× TBE	Tris 62.3 mM, Boric acid 62.3 mM, EDTA 1.4 mM
SHMG	Sucrose 250 mM, HEPES 1 mM, $MgCl_2$ 1 mM, Glycerol 10% (v/v), pH 7.0
Chloramphenicol MIC (*B. subtilis* 168)	10 µg/mL

Table S4. Primer Details for Colony and Template PCR

Primer Name	Sequence (5′→3′)	Target	Stock Conc	Working Conc	Amplicon Size	Supplier / Order No.
OptisehasA-F (hf)	GGCACAATCAGTGACAGGCA	*hasA* (forward)	100 pmol/µL	10 pmol/µL	500/1000 bp	Genefanavaran (203903)
OptisehasA-R (hr)	GCGGTTGCAGCACAAACAG	*hasA* (reverse)	100 pmol/µL	10 pmol/µL	500 bp	Genefanavaran (203903)
OptiNX02-R (nr)	AATCGCATCTGCATCCGCCA	NX02_04625 (reverse)	100 pmol/µL	10 pmol/µL	1000 bp	Genefanavaran (203903)

Table S5. Fermentation Medium Composition

Component	Concentration (g/L)
Sucrose	30
Yeast extract	10
Peptone (soybean)	5
$(NH_4)_2SO_4$	1
K_2HPO_4	9.15
KH_2PO_4	3
Trisodium citrate	1

Table S6. HA Titers in Single-Gene and Dual-Gene Strains

Strain	Time (h)	Replicate 1	Replicate 2	Mean ± SD
Single-gene	24	< LOQ	< LOQ	0.00 ± 0.00
Single-gene	48	0.54	0.44	0.49 ± 0.07
Single-gene	72	0.59	0.40	0.50 ± 0.13
Dual-gene	24	0.67	0.67	0.67 ± 0.00
Dual-gene	48	1.22	1.18	1.20 ± 0.03
Dual-gene	72	1.18	1.09	1.14 ± 0.07

- Unpaired two-tailed t-test for dual vs. single at each time point; paired t-test for 48 vs 72 h within each strain.

LOQ: limit of quantification (0.03 mg/mL).

Table S7. Raw HA concentrations (mg/mL) in wild-type and dual-gene recombinant *B. subtilis* strains, plus summary statistics and p-values (unpaired two-tailed t-tests vs. wild-type at each time).

Group	Replicate 1	Replicate 2	Replicate 3	Mean	SD	p-value (vs WT)
Recombinant, 24 h	0.19	0.20	0.25	0.21	0.03	< 0.05
Recombinant, 48 h	0.31	0.28	0.33	0.31	0.02	< 0.05
Wild-type, 24 h	0.08	0.01	0.00	0.04	0.04	—
Wild-type, 48 h	0.06	0.10	0.09	0.09	0.02	—

Notes:

- Data from CTAB turbidimetric assay (see Supplementary Protocol S8).
- Values are mean ± SD (n = 3).
- p-values are from unpaired two-tailed t-tests comparing recombinant vs. wild-type at the same time point.

- "—" denotes not applicable (no within-group time-point comparisons performed).

Table S8. Standard Calibration Data for HA Quantification

Serial dilutions of HA were prepared and analyzed using the CTAB turbidimetric assay. Absorbance was measured at 540 nm. The data below were used to generate the standard curve for HA quantification in experimental samples.

HA (mg/mL)	OD_{540}
1.00	0.79
0.50	0.55
0.25	0.33
0.12	0.16
0.06	0.07
0.03	0.02

Supplementary Table S8. HA standard curve data and regression parameters (n = 6). The coefficient of determination (R^2) calculated in Excel was approximately 0.95

Protocol S1. Plasmid reconstitution, dilution, storage, transformation, and ligation controls

- Reconstitution and dilution:
  - Resuspend lyophilized plasmid DNA (2–5 µg) in 30 µL sterile deionized water to 166.7 ng/µL; prepare 1:10 working dilution (16.67 ng/µL); aliquot and store at −20 °C.
- Preparation of chemically competent *E. coli* DH5α cells:
  Grow DH5α in LB medium (no antibiotic) at 37 °C with shaking until OD_{600} reaches 0.4–0.6. Chill culture on ice, centrifuge at 3500 rpm, 4 °C for 10 min. Wash cells twice with ice-cold 100 mM $CaCl_2$ in 15% glycerol. Resuspend final pellet in 1 mL $CaCl_2$/glycerol solution. Aliquot 100 µL per tube and store at −70 °C.
- Transformation into *E. coli* DH5α:
  - Add 6 µL diluted plasmid (16.67 ng/µL) to 100 µL chemically competent cells; incubate on ice 20 min; heat shock 42 °C for 60 s; recover on ice 2 min; add 900 µL antibiotic-free LB; incubate 1 h at 37 °C, 180 rpm. Plate 150–200 µL; pellet remaining 800 µL at 3500 rpm for 3 min, resuspend in 200 µL LB, plate; incubate overnight at 37 °C on LB + ampicillin (100 µg/mL).

- Ligation reactions and controls:
    - 15 µL ligation: 7 µL nuclease-free water, 5 µL digested plasmid DNA, 1.5 µL 10× T4 Ligase Buffer, 1.5 µL T4 DNA Ligase; add PEG equal to buffer volume to enhance ligation; incubate 16 °C for 2 h, then 22 °C for 2 h.
    - Negative control: identical mixture without ligase (8.5 µL water, 5 µL plasmid DNA, 1.5 µL ligase buffer, PEG) to confirm colonies depend on ligation.
    - Practical handling notes: dispense along tube wall; seal with parafilm; brief spins between steps.

Protocol S2. Enzymatic digestion, gel electrophoresis, and construct verification

- XbaI digestion:
    - 20 µL reaction: 9 µL nuclease-free water, 1 µL plasmid DNA (166.7 ng/µL), 2 µL Tango Buffer, 1 µL XbaI (10 U/µL); incubate 37 °C for 2 h.
- Gel electrophoresis and purification:
    - Resolve on 0.7% agarose; observe ~9 kb (backbone + *hasA*) and ~1.5 kb bands; excise backbone + *hasA* and purify per kit protocol.
- Construct size confirmation:
    - Dual-gene plasmid (pHT01-*hasA*- NX02_04625): 10,782 bp; single-gene variant (pHT01-*hasA*): 9,245 bp; size reduction ~1,537 bp corresponds to removal of the *tuaD* homolog segment including associated intergenic regions and tags.
- Gel/buffer preparation:
    - Prepare agarose gels and electrophoresis buffers per standard conditions (composition and run settings as used in this study).

Protocol S3. Agarose Gel Casting and Electrophoresis

1. Dissolve 0.7 g agarose powder in 100 mL 0.7× TBE buffer by microwave heating.
2. Allow solution to cool to ~60 °C, pour into casting tray with comb, and let solidify.
3. Assemble gel tank with 1 L 0.7× TBE buffer.
4. Load samples and run at standard voltage (5–8 V/cm) until adequate separation is achieved.
5. Document band sizes against a DNA ladder.

Protocol S4. *E. coli* Transformation and Plasmid Verification

1. Reconstitute lyophilized or gel-purified plasmid to 16.67 ng/µL in sterile water.
2. Add 6 µL plasmid to 100 µL chemically competent *E. coli* DH5α; incubate on ice 20 min.
3. Heat shock at 42 °C for 60 s; chill on ice 2 min.
4. Add 900 µL LB (no antibiotic); shake 1 h at 37 °C, 180 rpm.
5. Plate 150–200 µL on LB–ampicillin (100 µg/mL); optionally pellet and resuspend remaining culture for additional plating.
6. Incubate overnight at 37 °C.
7. Screen colonies by PCR with *hasA* specific primers.

Protocol S5. *Bacillus subtilis* Electroporation and Screening

1. Electrocompetent Cell Preparation

a. Inoculate *B. subtilis* 168 into LBSP; grow to $OD_{600} \approx 1.0$ at 37 °C, 180 rpm.
b. Chill cultures on ice; pellet at 10,000×g, 5 min, 4 °C; wash three times with ice-cold SHMG; resuspend in SHMG; aliquot 500 µL; store at −80 °C.

2. Electroporation

a. Thaw 500 µL competent cells; add 100 µL cells + 10 µL plasmid DNA (0.01–1 µg) into a pre-chilled 0.2 cm cuvette.
b. Pulse at 2,500 V (12.5 kV/cm), 25 µF, ∞ Ω.
c. Immediately add 4.5 mL room-temperature LBSPG; shake at 37 °C for 1–3 h.

3. Recovery and Selection

a. Plate 100 µL directly and pellet/resuspend remaining culture for additional plating on LB–chloramphenicol (10 µg/mL).
b. Incubate statically at 37 °C for 12–16 h.

4. Colony Processing

a. Streak individual colonies on fresh LB–chloramphenicol; incubate overnight.
b. Prepare 25% glycerol stocks; store at −80 °C.
c. Pick colonies for PCR template.

Protocol S6. Colony and Plasmid PCR Protocols

1. Primer reconstitution
    - Centrifuge lyophilized primers 30 s. Reconstitute to 100 pmol/µL in injection-grade water. Prepare 10 pmol/µL working solutions (5 µL stock + 45 µL water). Store at −20 °C.
2. Template preparation
    - **Colony PCR**: Transfer minimal biomass to 10 µL sterile water; lyse by five cycles of 95 °C/10 min and 0 °C/5 min.
3. Reaction setup (10 µL total)
    - 1 µL template (colony lysate or plasmid)
    - 0.5 µL forward primer (10 µM)
    - 0.5 µL reverse primer (10 µM)
    - 5 µL 2× Taq Master Mix
    - q.s. to 10 µL with sterile water
4. Thermal cycler programs
    - **Short amplicon (~500 bp)**
      • 95 °C/10 min; 30× (95 °C/30 s, 51 °C/45 s, 72 °C/50 s); 72 °C/10 min
    - **Long amplicon (~1000 bp)**
      • 95 °C/10 min; 30× (95 °C/30 s, 56.1 °C/54 s, 72 °C/1 min); 72 °C/10 min
5. Analysis
    - Run PCR products on 0.7% agarose in 0.7× TBE at 5 V/cm. Visualize expected bands: 500 bp, 1000 bp.

Protocol S7. HA Purification

1. **TCA Precipitation**
    - To 1 mL clarified culture supernatant, add 100 µL of 100 % (w/v) TCA.
    - Incubate on ice for 30 min.
    - Centrifuge at 13230 × g for 15 min at 4 °C.
    - Carefully decant the supernatant.
2. **Ethanol Precipitation**
    - Add 2 mL cold 100 % ethanol to the pellet; mix by gentle inversion.
    - Freeze at –70 °C for 2 h.
    - Centrifuge at 6 000 × g for 20 min at 4 °C; discard supernatant.
    - Wash pellet with 1 mL cold 100 % ethanol; incubate at –20 °C for 1 h.
    - Centrifuge again at 6 000 × g for 20 min at 4 °C; remove ethanol and air-dry pellet briefly.
3. **Resuspension**

- Dissolve pellet in 1 mL deionized water.
- Incubate at 45 °C (shaker at ~220 rpm, tubes angled ~45°) until fully dissolved.
- Store at 4 °C until assay.

Protocol S8. CTAB Turbidimetric Quantification

1. **Assay setup**
    - In a sterile 96-well plate, pipette 75 µL sample + 75 µL acetate buffer (0.2 M, pH 6).
    - Pre-warm CTAB reagent (2.5 % w/v in 0.5 M NaOH) at 37 °C; add 150 µL to each well.
    - Immediately mix gently to prevent bubble formation
2. **Absorbance measurement**
    - Read Abs_{540} within 2–3 min of CTAB addition (to prevent turbidity decay).
    - Microplate reader settings (Gen5 3.03):
      • New run → At runtime → Read → λ = 540 nm → Well selection → Read.
      • Use consistent plate layout; blank wells contain 75 µL water + 225 µL buffer/CTAB.
3. **Calibration curve**

- Prepare HA standards from 1 mg/mL stock by serial 1:1 dilutions to 0.006 mg/mL.
- Excel: column A = concentration; column B = Abs_{540}. Insert → Scatter → Scatter with smooth lines & markers → Quick Layout 9 → display equation & R^2. Apply equation immediately to sample Abs_{540} values to calculate HA concentration.
- HA standard: Hyaluronic acid sodium salt from *Streptococcus equi*, Sigma-Aldrich, Lot# BCBN4845V.

Protocol S9. FTIR Spectroscopy

1. **Sample Preparation**

KBr pellet mode: KBr powder was ground and sieved to remove coarse particles. Dried HA was weighed to 1 wt % relative to KBr and mixed in an agate mortar and pestle until homogeneous. Approximately 200 mg of the blend was transferred into a stainless-steel die and pressed under vacuum (or in a dry-air atmosphere) at ~10 000 t/cm² to form a transparent disc.

2. **Instrument Settings and Data Processing**

Wavenumber range: 4000–400 cm^{-1}

Resolution: 4 cm^{-1}

Background correction: Applied before each measurement

Post-acquisition processing: All spectra underwent baseline correction and peak analysis to characterize absorption features

3. **Key Absorption Bands**

~3400 cm^{-1}: O–H stretching

~1600 cm^{-1}: C=O stretching (carboxyl group)

~1550 cm^{-1}: N–H bending (amide II)

~1050 cm^{-1}: C–O–C stretching (glycosidic linkage)

Protocol S10. GPC Analysis

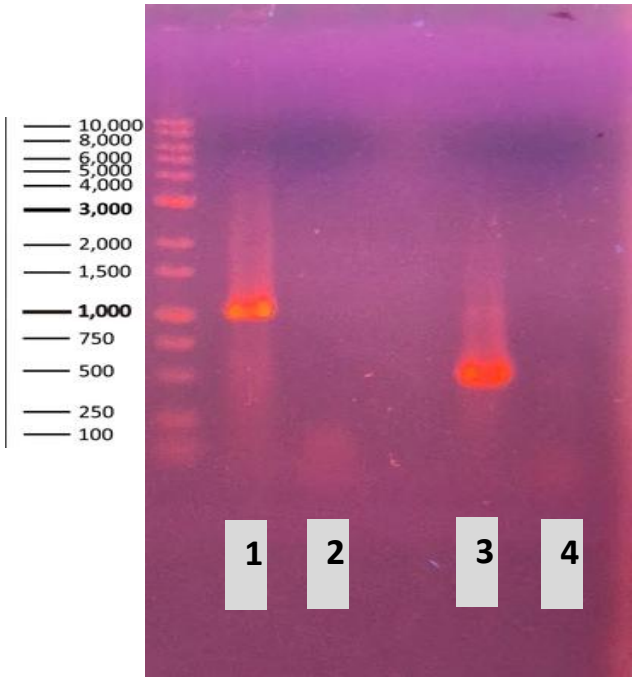
1. **Instrumentation**
    - Two PL aquagel-OH mixed-H columns (300 × 7.5 mm, 8 µm)
    - Separation range: 6×10^3 to 1×10^7 g/mol
    - Mobile phase: H_2O
    - Flow rate: 1.0 mL/min
    - Column temperature: 35 °C
    - Detector: Refractive Index (RI)
2. **Sample preparation**
    - HA samples filtered through 0.45 µm membranes
    - Injection volume: 100 µL
3. **Calibration**
    - PEG standards used for calibration
    - Molecular weight range: 1.18×10^6 to 1030 g/mol
    - Calibration curve constructed using log(MW) vs. retention time
4. **Data analysis**

- Weight-average molecular weight (Mw) and polydispersity index (PDI) calculated using standard GPC software.

Supplementary Figure S1. Growth of *E. coli* DH5α transformants on LB–ampicillin

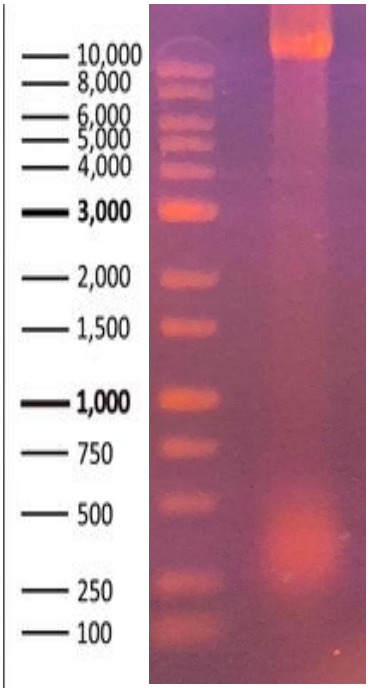
Supplementary Figure S1. Representative LB–ampicillin plate showing discrete *E. coli* DH5α colonies after heat-shock transformation with PST. Transformation protocol is described in Supplementary Protocol S1. Mock transformations without plasmid DNA resulted in no colonies.

Supplementary figure S2



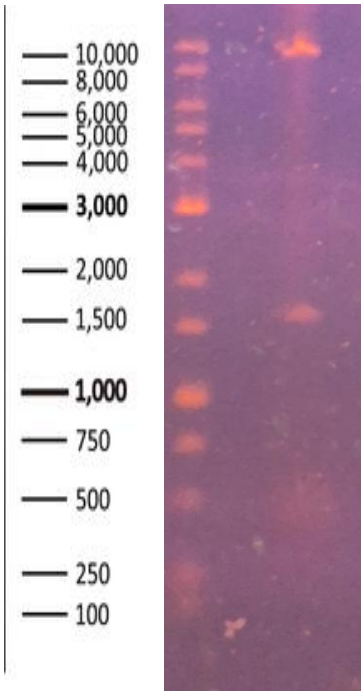
Supplementary Figure S2. PCR validation of synthesized pHT01-*hasA*–NX02_04625 construct. Lane 1: *hasA*–NX02_04625 (~1000 bp), Lane 2: no-template control, Lane 3: *hasA* (~540 bp), Lane 4: no-template control. Primer sequences and PCR conditions are listed in Supplementary Table S4 and Protocol S6.

Supplementary Figure S3



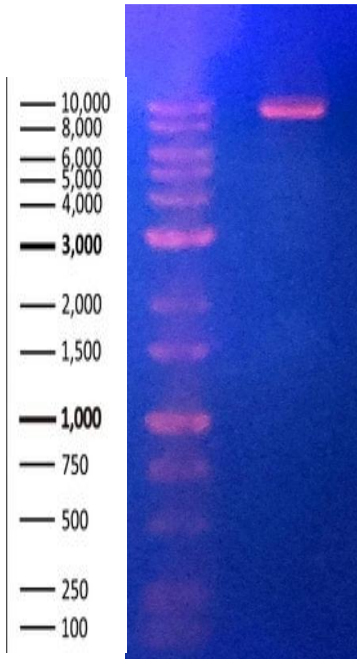
Supplementary Figure S3. Agarose gel electrophoresis of pHT01-*hasA*–NX02_04625 extracted from *E. coli* DH5α. A single band at approximately 10.8 kb confirms plasmid integrity prior to removal of the NX02_04625 coding sequence. Gel conditions: 0.7% agarose in 0.7× TBE buffer, run at 100 V for 45 min; DNA visualized under UV after ethidium bromide staining.

Supplementary Figure S4.



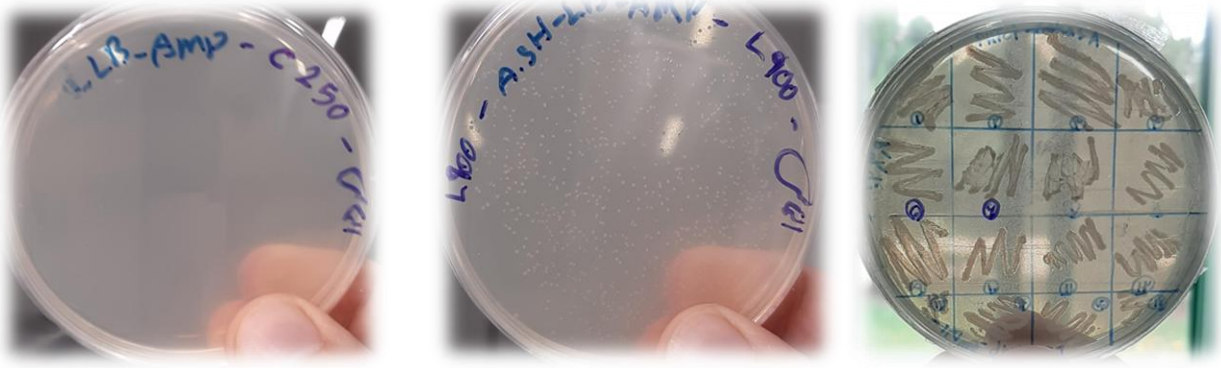
Supplementary Figure S4. Agarose gel electrophoresis of XbaI-digested pHT01-*hasA*–NX02_04625. Two distinct bands were observed at 9 kb and ~1.5 kb, corresponding to the plasmid backbone containing the *hasA* and the excised NX02_04625 fragment. Gel conditions**:** 0.7% agarose in 0.7× TBE buffer, run at 100 V for 45 min; DNA visualized under UV after ethidium bromide staining.

Supplementary Figure S5



Supplementary Figure S5. Agarose gel electrophoresis of the recycled pHT01-*hasA* following XbaI digestion and gel extraction. A single band at ~9.2 kb confirms successful recovery of the vector backbone containing the *hasA* gene for single-gene construct assembly. The gel (0.7% agarose in 0.7× TBE buffer) was run at 100 V for 45 min and stained with ethidium bromide; DNA was visualized under UV illumination.

Supplementary Figure S6.



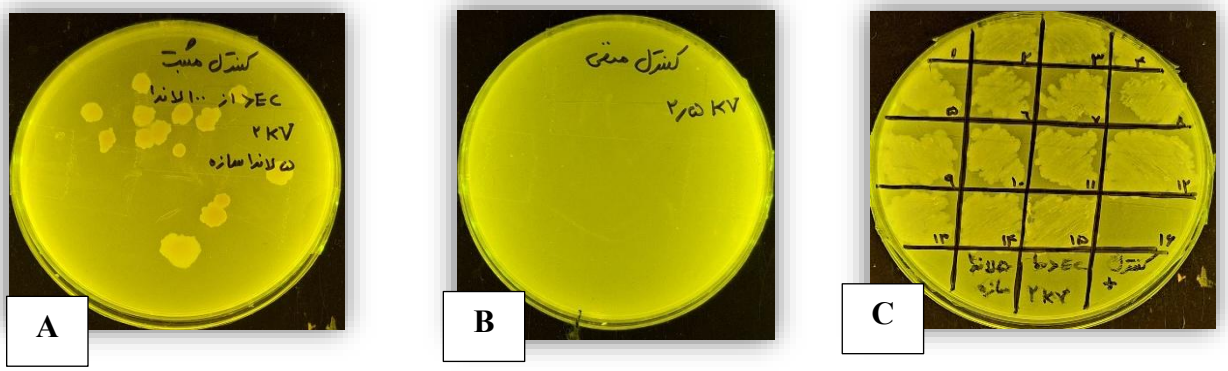
A **B** **C**

Supplementary Figure S6. Representative plate images from the transformation assay:

- **A**: LB–Amp plate with linear construct (no colonies).
- **B**: LB–Amp plate with ligated construct (numerous colonies).
- **C**: Expanded view of colonies after overnight incubation.

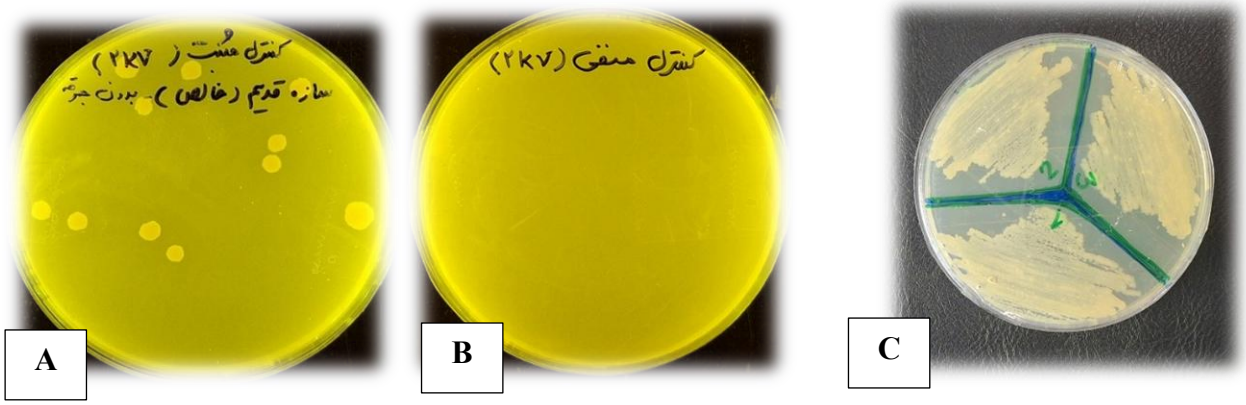
Plate conditions: LB agar + 100 µg/mL ampicillin; incubation at 37 °C for 16–18 h.

Supplementary Figure S7.



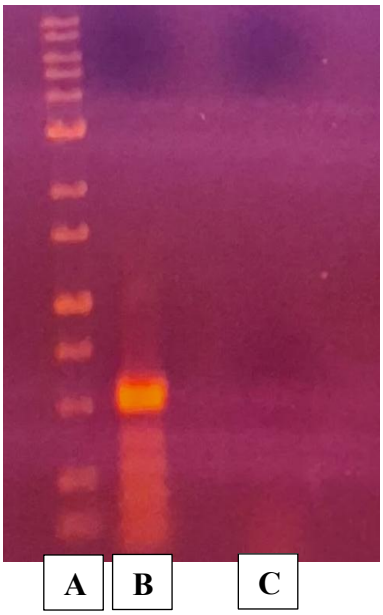
Supplementary Figure S7. Growth of *Bacillus subtilis* 168 on LB–chloramphenicol plates following electroporation with the single-gene construct (pHT01-*hasA*). (A) Transformed cells showing distinct colony formation on selective medium, indicating successful uptake and expression of the plasmid. (B) Negative control plate (electroporated cells without plasmid DNA), showing no growth under identical conditions, confirming the specificity of antibiotic selection. (C) Re-plated transformed colonies on fresh LB–chloramphenicol plates, demonstrating stable propagation and plasmid maintenance.

Supplementary Figure S8.



Supplementary Figure S8. Colony formation of *Bacillus subtilis* 168 on LB–chloramphenicol plates following electroporation with the dual-gene construct (pHT01-*hasA*–NX02_04625). (A) Transformants showing distinct colonies, confirming successful uptake and expression. (B) Negative control (no plasmid DNA), showing no growth and validating selection specificity. (C) Re-plated transformants on fresh LB–chloramphenicol, demonstrating stable propagation and plasmid maintenance.

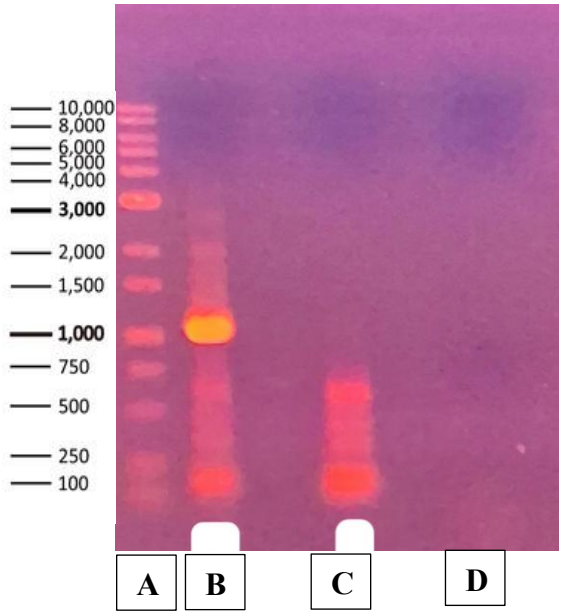
Supplementary Figure S9



Supplementary Figure S9. Agarose gel electrophoresis of colony PCR products:

- Lane A: 1000 bp ladder
- Lane B: pHT01-*hasA* transformant, 540 bp band
- Lane C: Negative control, electrocompetent cells electroporated without plasmid DNA.

Supplementary Figure S10

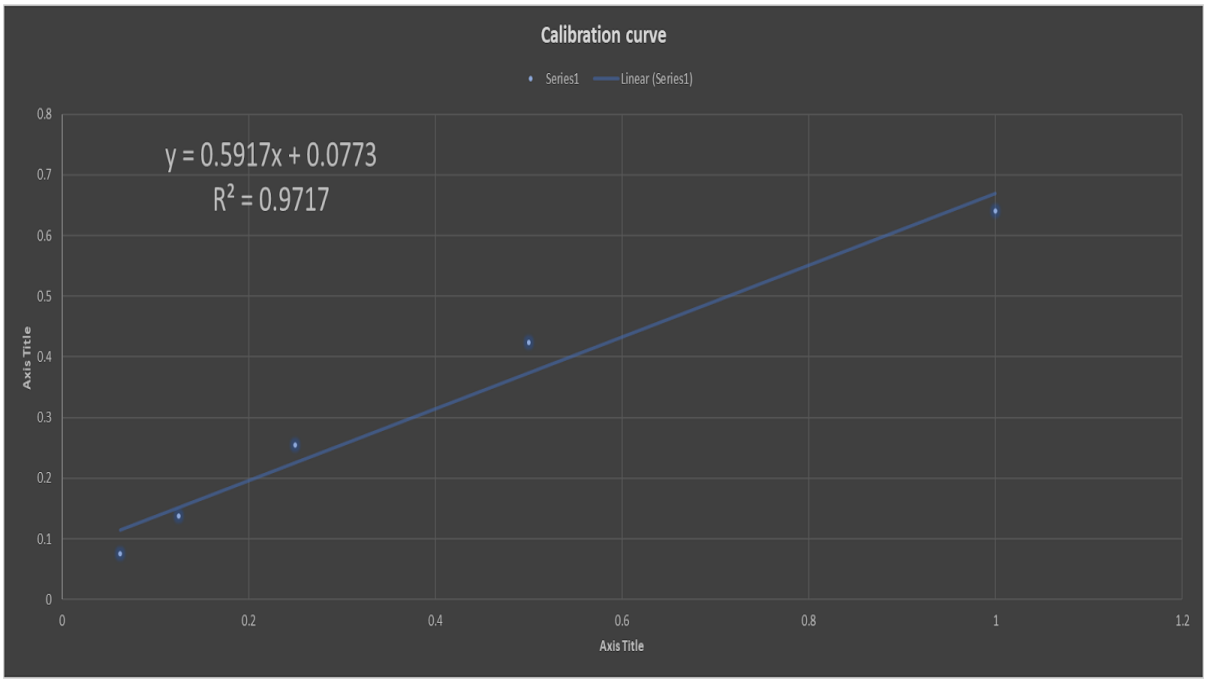


Supplementary Figure S10 . Representative agarose gel of colony PCR products:

- Lane A: 1000 bp DNA ladder
- Lane B: pHT01-*hasA*–NX02_04625 transformant, 1 000 bp band
- Lane C: Negative control colony, electrocompetent cells electroporated without plasmid DNA.
- Lane D: No-template control (NTC), no amplification

Supplementary Figure S11

[HA] (mg/mL)	Abs_{540} Rep 1	Abs_{540} Rep 2	Abs_{540} Rep 3	Mean Abs_{540}
1.00	0.63	0.63	0.64	0.64
0.50	0.42	0.41	0.44	0.42
0.25	0.24	0.28	0.24	0.25
0.12	0.13	0.13	0.14	0.13
0.06	0.07	0.07	0.07	0.07
0.03	0.03	0.05	0.04	0.04
0.01	0.02	0.01	0.01	0.01
0.00	0.00	0.00	0.00	0.00



425

426 **Supplementary Figure S11.** Calibration Curve for CTAB Turbidimetric HA Assay

427 The coefficient of determination (R^2) calculated in Excel was 0.97