HAYATI
Journal of Biosciences

Vol. 32 No. 1, January 2025 155-163
DOI:10.4308/hjb.32.1.155-163
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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Cloning and Optimized Expression of Bst DNA Polymerase from *Geobacillus stearothermophillus* in *Escherichia coli* BL21

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ARTICLE INFO

Article history:
Received August 30, 2024
Received in revised form September 19, 2024
Accepted September 24, 2024

KEYWORDS:
Bst DNA Polymerase,
Nucleic Acid Amplification Method,
LAMP.

ABSTRACT

Bst DNA polymerase possesses strand displacement activity, enabling isothermal DNA amplification without requiring a thermal cycler. This enzyme is utilized in the Loop-Mediated Isothermal Amplification (LAMP) method, which offers advantages in speed and simplicity over Polymerase Chain Reaction (PCR) method. The growing demand for Bst DNA polymerase highlights the need for cost-effective in-house production, as a commercial option is economically challenging. For that purpose, this study aims to construct and optimize the expression of Bst DNA polymerase from *Geobacillus stearothermophilus* in *Escherichia coli*. The expression constructs pET16b.BstHF vector was constructed using Gibson Assembly and expressed in *E. coli* BL21 (DE3). Optimal expression was achieved with 1 mM IPTG, induction at OD_{600} 0.8 and 6-hour induction time. The purified enzyme was achieved with a protein yield of 2,175 mg/L culture and demonstrated effective polymerase activity for LAMP.

1. Introduction

Nucleic acid amplification (NAA) is widely used in fields such as pathogen diagnostics, genetic analysis, gene cloning, recombinant protein production, and halal product authentication (Widyanto *et al*. 2021; Garg *et al*. 2022; Muflihah *et al*. 2023; Kundu *et al*. 2024). Nucleic acid amplification methods can be categorized into thermocycling and isothermal amplification (Atceken *et al*. 2023). The Polymerase Chain Reaction (PCR) is the most widely used thermocycling amplification method since it was introduced by Kary Mullis in 1983 (Mullis 1990; Bu *et al*. 2023). PCR is extensively applied in clinical applications, disease diagnosis, gene cloning and sequencing, and rapid and highly sensitive quantitative and genomic studies (Garibyan & Avashia 2013). However, the PCR technique has limitations, such as the requirement for a thermal cycler limiting its application in resource-limited settings (Srivastava & Prasad 2023). Thus, alternative amplification methods are necessary to address these limitations (Zanoli & Spoto 2013).

Isothermal amplification methods offer advantages over PCR, including faster amplification times and the ability to operate at a constant temperature, removing the need for a thermal cycler (Zhao *et al*. 2015; Oliveira *et al.* 2021; Boonbanjong *et al*. 2022). Several isothermal amplification methods have been developed, including Recombinase Polymerase Amplification (RPA), Nucleic Acid Sequence-Based Amplification (NASBA), Rolling Circle Amplification (RCA) Strand Displacement Amplification (SDA), Helicase Dependent Amplification (HDA), Loop-Mediated Isothermal Amplification (LAMP) (Lobato & O'Sullivan 2018; Garafutdinov *et al*. 2021 Boonbanjong *et al*. 2022; Zhang *et al*. 2024).

The LAMP introduced by Notomi *et al*. in 2000 is notable for its high specificity, efficiency, and rapid amplification (Wong *et al*. 2018; Boonbanjong *et al*. 2022; Deliveyne *et al*. 2023). This technique significantly reduces amplification time (Notomi *et al*. 2000, 2015). LAMP can be integrated with

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colorimetric and turbidity-based detection methods, enhancing sample identification and establishing it as a rapid, simple, and cost-effective molecular detection technique for field use (Garg *et al*. 2022; Yang *et al*. 2024). The LAMP method utilizes DNA polymerase enzymes with high strand displacement activity, with Bst DNA polymerase being the most commonly used enzyme in this method (Soroka *et al*. 2021). The demand for Bst DNA polymerase is expected to increase due to its potential for rapid and easy application. However, Bst DNA polymerase remains costly in Indonesia due to reliance on commercial imports, and research in this area is limited (Agustriana *et al*. 2023). For that purpose, this study aims to construct and optimize the production of Bst DNA polymerase high fidelity (BstHF).

2. Materials and Methods

2.1. Plasmids, Bacterial Strains, and Culture Media

This study used *Escherichia coli* BL21 (DE3) as the expression host. The Bst DNA polymerase gene insert fragment was obtained from the pOpen BSTHF vector (FreeGenes, USA), while the vector backbone fragment from pET16b.PFU (Addgene, USA). The medium used in this study was Luria-Bertani (LB) (Himedia, Maharashtra, India) supplemented with 100 ppm ampicillin. The protein production of BstHF was induced using Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) (Thermo Scientific, USA).

2.2. Cloning pET16b.BstHF Expression Vector using Gibson Assembly

The cloning of the pET16b.BstHF expression vector was carried out using the Gibson assembly method (Gibson *et al.* 2009). Amplification of the BstHF insert (1806 bp) from pOpenBstHF and the vector backbone (5649 bp) from pET16b.PFU using PCR method. The primers used for the amplification of the BstHF insert fragment and the pET16b vector backbone are listed in (Table 1). The overlapping regions between the insert and the vector were created using the primer sequences, which are indicated by underlining. The Gibson Assembly method utilized Gibson Assembly® Master Mix (New England Biolabs Inc., USA), resulting in the pET16b.BSTHF expression vector (7419 bp). This vector features an IPTG-inducible T7 promoter, an ampicillin antibiotic resistance marker, a 10x His tag, a Factor Xa cleavage site, and the BstHF DNA polymerase gene (Figure 1). This construct was subsequently transformed into *Escherichia coli* BL21 (DE3) via the heat shock method (Hanahan 1983). The success of the transformation was validated using PCR and Sanger sequencing, with primers listed in (Table 1).

2.3. Optimization of BstHF DNA Polymerase Protein Production

A single colony from the master plate was inoculated into 10 ml Luria Bertani (LB)-ampicillin (100 µg/ml) and incubated for 16 hours (37°C, 150 rpm). Subsequently, 1% of the overnight culture was inoculated into 50 ml LB-ampicillin (100 µg/ml) in a 250 ml Erlenmeyer flask and incubated (37°C, 150 rpm) until OD_{600} 0.6 Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 0, 0.2, 0.4, 0.8, and 1 mM concentrations. The cultures were then incubated for 6 hours at 37°C with shaking at 150 rpm. Harvesting by centrifugation at 8,000 rpm for 6 minutes at 4°C. The pellet was resuspended in lysis buffer (25 mM Tris-HCl, supplemented with 1 mM PMSF) at a ratio of 1:10 and sonicated on ice with 30s ON and 15s OFF cycles for 6 minutes at 40% amplitude. The lysate was then centrifuged at 16,000 x g for 30 minutes at 4°C. The supernatant, representing the soluble fraction, was subjected to protein concentration measurement using the Bradford Assay. The soluble fraction was visualized using 12.5% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), followed by BstHF protein percentage (%) determination using ImageJ software. The percentage of BstHF protein was then compared with the total target protein concentration (mg/L culture). Each treatment was conducted in triplicate. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test. Growth curve analysis was also performed using a spectrophotometer at a wavelength of 600 nm. Sampling points were taken every hour following IPTG induction. The optimal point for IPTG induction was selected based on the highest BstHF protein concentration while ensuring robust *E. coli* growth.

The optimal IPTG concentration was used to optimize the IPTG induction point with varying OD_{600} values of 0.4, 0.6, 0.8, and 1.1. The optimal induction OD_{600} point was then used to optimize induction duration, with 3, 6, 12, and 16 hours being evaluated in this study.

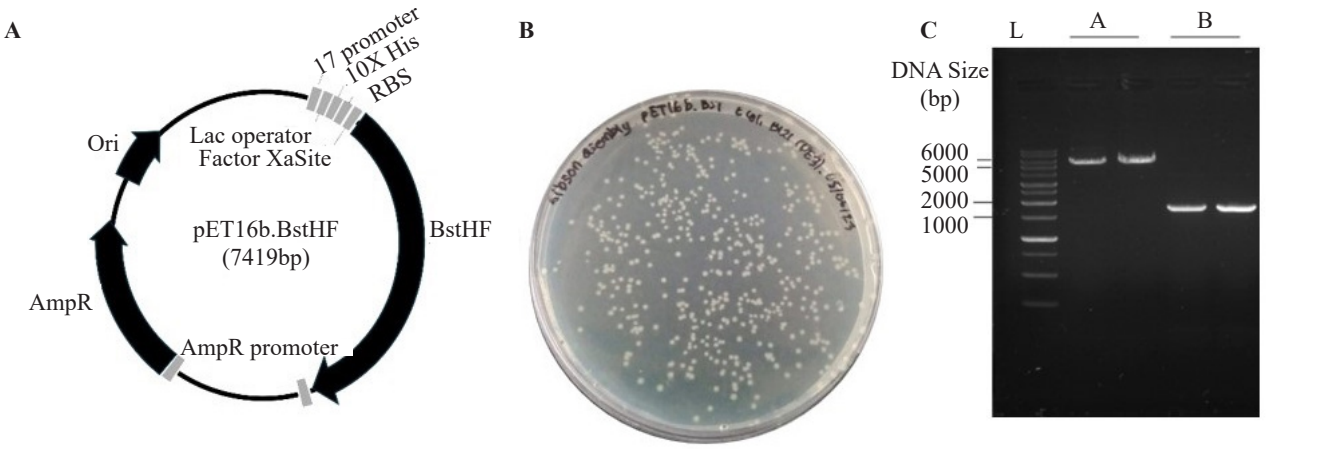

Figure 1. (A) Plasmid map of pET16b.BstHF (B) Screening plate of the *E. coli* BL21 (DE3) transformant (C) PCR for validation of transformation using vector-specific primers (A = 5649 bp) and insert-specific primers (B =1806 bp)

Table 1. Primers for target gene amplification

Primers	Nucleotide sequence (5'-3')	Amplicon target (bp)
pET16b.PFU Vektor Forward	CCGCTGAGCAATAACTA GCATAACCCC	5649
pET16b.PFU Vektor Reverse	ATGACGACCTTCGATATG GCCGC	
pOpen BstHF Insert Forward	CATATCGAAGGTCGTCAT ATGGAGGCCAAAGGC GAAAAGC	1806
pOpen BstHF Insert Reverse	CTAGTTATTGCTCAGC GGTCACTTGGC GTCGTACCAAG	

2.4. Bst DNA Polymerase Purification using Ni-NTA Affinity Chromatography

Purification was done using affinity chromatography with a Nickel-Nitrilotriacetic Acid (Ni-NTA) resin column (Thermo Scientific, cat#: 88221). The elution fraction was then measured using the Bradford Assay to determine the concentration of the purified BstHF protein. The eluted protein from the purification process underwent ultrafiltration treatment. The ultrafiltrated samples were then subjected to dialysis for 16 hours in dialysis buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol). The dialyzed samples were subsequently stored in a storage buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% glycerol, 0.1% Triton X-100). The LAMP method used the dialyzed sample in the activity assay stage. Visualization of crude fraction samples from BstHF protein production and dialyzed fraction samples was performed using SDS-PAGE.

2.5. Activity Assay using Loop-Mediated Isothermal Amplification (LAMP)

The activity of BstHF DNA polymerase was assessed using the LAMP method. The LAMP reaction mixture consisted of 10x isothermal buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X), 5 mM $MgSO_4$, 400 mM betaine, 300 µM each dNTP, 6 primers (0.8 µM F3; 0.8 µM B3; 2 µM FIP; 2 µM BIP; 1.6 µM LoopF and 1.6 µM LoopB), and Nuclease-Free Water (NFW), in a total reaction volume of 25 µL. Bst DNA polymerase Large Fragment (New England Biolabs, cat # M0275S) was a positive control. The results of the LAMP amplification were then observed using agarose gel electrophoresis and colorimetric method with an intercalating dye, Diamond™ Nucleic Acid, at 1 µL. Amplification was indicated by the presence of ladder-like patterns in the agarose gel electrophoresis results and fluorescence in the colorimetric method.

3. Results

3.1. Cloning and Optimization of BSTHF DNA Polymerase Protein Production

The expression vector map pET16b.BstHF is presented in (Figure 1A). Transformation of the pET16b.BstHF expression vector into *Escherichia coli* BL21 (DE3) via Gibson assembly yielded transformant colonies (Figure 1B). The success of the transformation was confirmed through PCR and sequencing, validating both the transformation and the construction of the expression vector (Figure 1C).

Optimization of IPTG concentration revealed that 1 mM IPTG resulted in the highest average concentration of BstHF protein at 0.317 mg/L culture, which was significantly different from other IPTG concentrations (0, 0.2, 0.4, 0.8 mM) (Figure 2A). Analysis of BstHF protein concentration during IPTG induction point at varying Optical Densities (OD_{600}) (Figure 2B) indicated that IPTG induction at OD_{600} 0.8 produced the highest average concentration of 0.3850 mg/L culture. This significantly differed from others (0.4, 0.6, and 1.1) with protein BstHF concentrations of 0.09, 0.20, and 0.34 mg/L, respectively. Growth curve analysis (Figure 2D) showed that OD_{600} 0.8 corresponds to the mid-logarithmic phase.

Protein concentration analysis for IPTG induction duration (Figure 2C) demonstrated that BstHF protein concentration increased with longer IPTG induction durations. The highest average concentration of 0.4228 mg/L culture was achieved at a 16-hour induction, although this was not significantly different from 6-hour (0.3786 mg/L culture) and 12-hour (0.3942 mg/L culture) inductions. The lowest average concentration of BstHF protein, 0.1545 mg/L culture, was observed at a 3-hour induction. The optimal conditions for producing Bst DNA polymerase were identified as 1 mM IPTG induction, induction at OD_{600} of 0.8, followed by a 6-hour induction period.

3.2. Purification and Activity Assay

The BstHF DNA polymerase was purified using Ni2+-nitrilotriacetic acid (Ni-NTA) resin. The protein concentration obtained was 2,175 mg/L culture. Post-purification, the eluate was concentrated by ultrafiltration and dialyzed overnight. SDS-PAGE visualization of the dialyzed samples is shown in (Figure 3).

Amplification was performed according to Kanchanaphum & Maneenin (2014). Gel electrophoresis visualization of amplification products

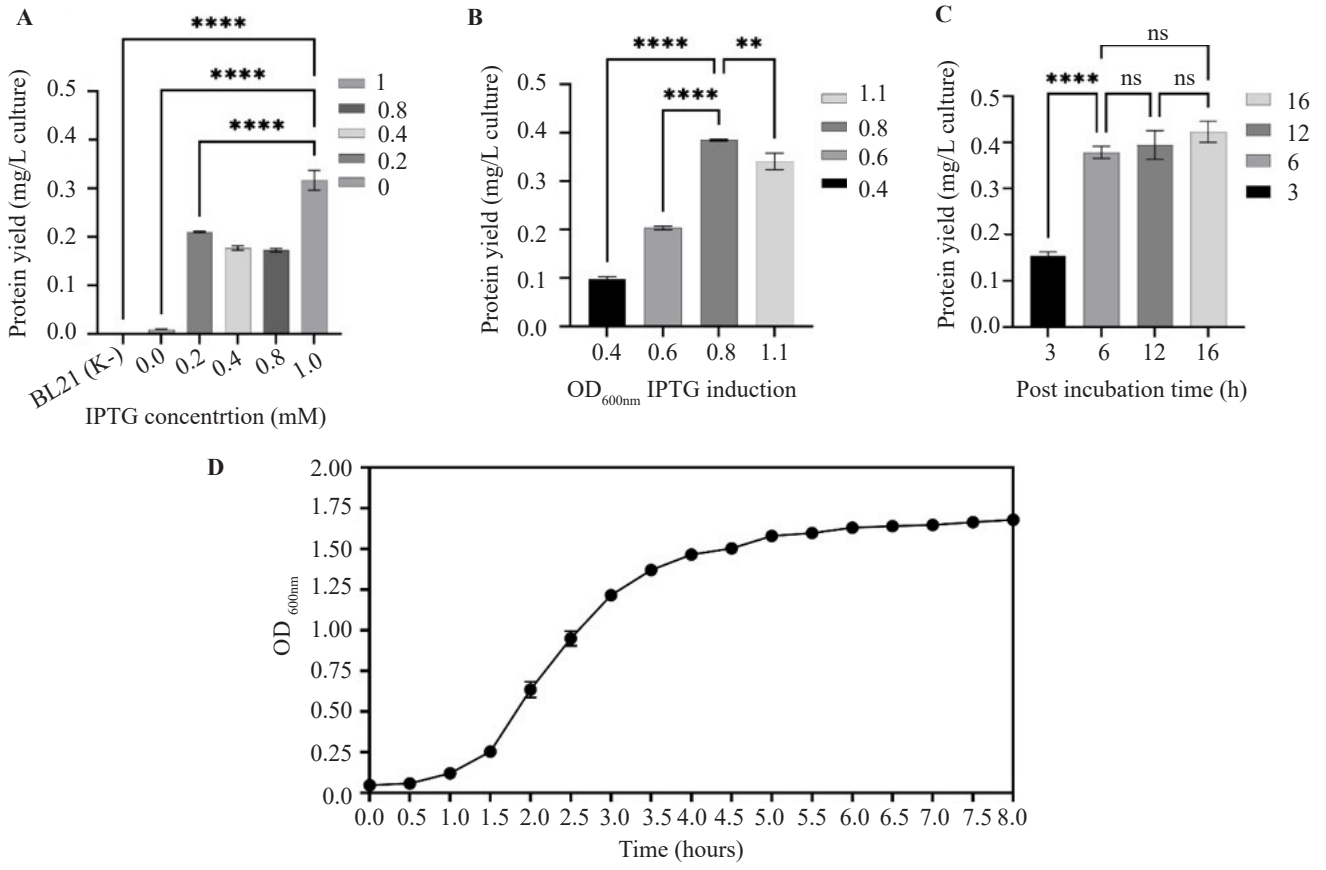

Figure 2. (A) Protein yield (mg/L culture) under variation of IPTG concentration (B) Protein yield (mg/L culture) (C) Protein yield (mg/L culture) under variation of IPTG induction duration under variation of IPTG induction point (D) Growth curve of *E. coli* BL21. BstHF in LB medium+ampicillin 100 ppm, at 37°C, 150 rpm (triplicate). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test with a significance level of p-value of < 0.0001 (****), < 0.002 (**), > 0.05 (ns)

is shown in (Figure 4A). LAMP results exhibited a ladder-like pattern due to the formation of multiple dumbbell structures (Notomi *et al*. 2015).

Amplification products from 2 and 3 µL (protein concentrations 0.079 µg/µL) of BstHF DNA polymerase dialyzed sample and commercial Bst DNA polymerase (C+) are shown a ladder-like pattern. Colorimetric analysis (Figure 4B) further confirmed successful amplification with both BstHF and commercial Bst DNA polymerase, as indicated by fluorescence in the 2 and 3 µL samples. Diamond Nucleic Acid binds to the external groove of DNA, producing fluorescence proportional to the amount of amplification (Haines *et al*. 2015). The visualization results revealed a ladder-like band pattern and fluorescence in the colorimetric assay for all activity test samples. This confirms the successful amplification by the LAMP method and indicates the polymerase activity of the BstHF DNA Polymerase enzyme.

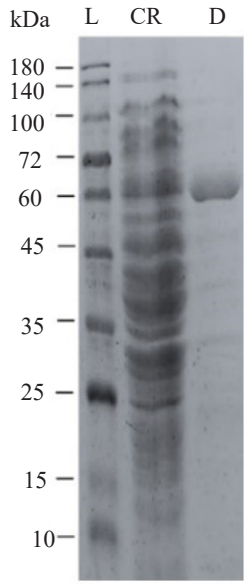

Figure 3. SDS-PAGE of dialyzed samples (L: Ladder, CR: Crude, D: Dialyzed sample)

4. Discussion

The Bst DNA polymerase enzyme used in this study was obtained from Freegenes, Stanford. The BstHF DNA polymerase gene, consisting of 1770 bp nucleotides, encodes a 543 amino acid protein with an estimated molecular weight of 66.8 kDa. This enzyme, a large fragment of the DNA polymerase I from *Geobacillus stearothermophilus*, exhibits polymerase activity at 50°C. The large fragment lacks the N-terminal domain, specifically the 5'-3' exonuclease activity, resulting in the enzyme devoid of 5'-3' exonuclease activity (Oscorbin & Filipenko 2023). This truncated polymerase, often referred to as BF or Bst polymerase, denotes a shortened form of the original enzyme (Oscorbin & Filipenko 2023).

In this study, the optimal IPTG concentration for BstHF DNA polymerase production was 1 mM. Studies by (Ma *et al.* 2016; Oscorbin *et al*. 2017; Agustriana

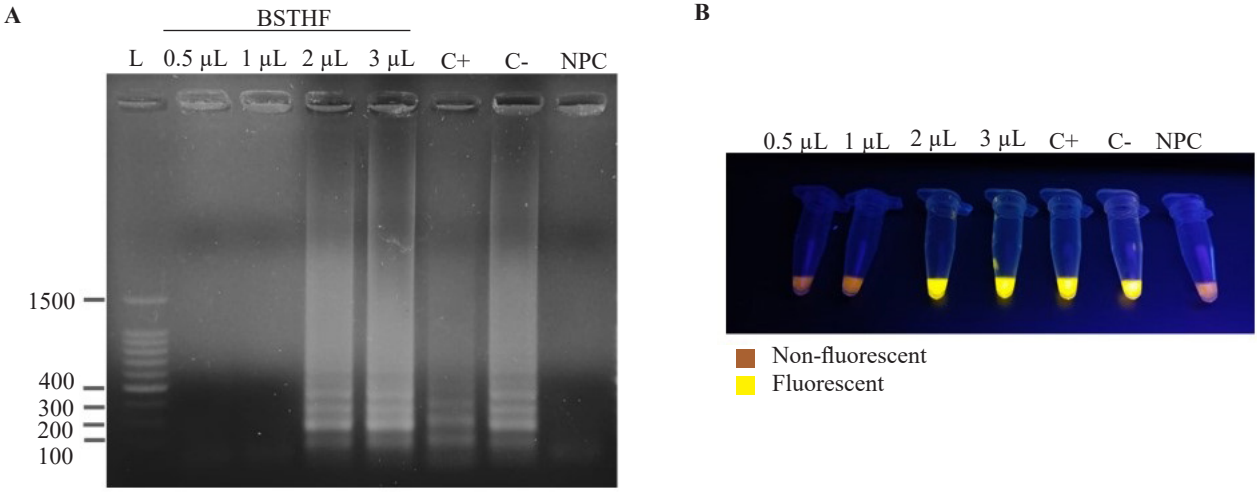

Figure. 4 (A) Elektroforegram LAMP amplification results (B) Colorimetric method of LAMP (L: Ladder, C+: positive Control (using target DNA samples and LAMP amplification with commercial Bst polymerase enzyme), C-: negative control (using non-target DNA samples and LAMP amplification with BstHF enzyme produced in this study), NPC: Non-Protein Control (using target DNA samples and LAMP amplification without the addition of Bst enzyme)

et al. 2023) also indicate that 1 mM IPTG is optimal for producing the highest concentration of Bst DNA polymerase. Research by (Agustriana *et al*. 2023) demonstrated that increasing IPTG concentration beyond 1 mM does not lead to higher recombinant protein concentrations. Further research by (Dvorak *et al*. 2015) indicates that IPTG can be toxic to *E. coli* BL21 (DE3), exacerbating haloalkane substrate toxicity and causing significant damage to the host *E. coli* BL21 (DE3). Therefore, a lower IPTG concentration for induction is preferred as it can provide cost efficiency and avoid metabolic burden (Donovan *et al*. 1996).

The highest BstHF protein concentration was achieved with IPTG induction at an $OD_{600\ nm}$ of 0.8, corresponding to the mid-log phase. This finding aligns with the literature where Rajacharya *et al.* (2024) demonstrated that induction during the mid-log phase yields higher growth rates and protein concentrations than early-log induction. The mid-log phase is optimal for protein synthesis due to the availability of cellular resources (Rosano & Ceccarelli 2014; Rajacharya *et al*. 2024). Induction during the mid-log phase is commonly considered optimal for recombinant protein production (Xu *et al*. 2006). Variation in IPTG induction time OD_{600} for protein Bst DNA polymerase induction was studied (Agustriana *et al.* 2023) found an optimal OD_{600} of 1.1, whereas Oscorbin *et al*. (2015), Ma *et al.* (2016) and Li *et al*. (2017) identified an OD_{600} of 0.6 as the optimal point for IPTG induction.

The optimal IPTG induction duration for BstHF production was 6 hours. This duration provided a high average protein concentration without significant differences compared to the highest concentration observed, thus allowing for faster production. Emami (2020) also identified 6 hours as the optimal IPTG induction duration. Similarly, Agustriana (2023) found 6 hours to yield the highest average concentration of Bst DNA polymerase. Using a rhamnose-based promoter system, Laksmi *et al* (2023) also identified 6 hours as the optimal induction duration for Bst DNA polymerase production. Prolonged IPTG induction can lead to metabolic stress and decreased efficiency in *E. coli* (Azaman *et al*. 2010).

The purified BstHF protein concentration achieved in this study was 2.175 mg/L of culture, which is lower compared to Agustriana *et al*. (2023), who reported 11.7 mg/L culture with optimized parameters, including IPTG concentration, OD_{600} induction point, IPTG induction duration, and agitation. Li *et al* (2017) reported 1.5 mg/L culture, while Alekseenko *et al.* (2021) achieved 3 mg/L culture.

Activity assays demonstrated that the BstHF DNA polymerase enzyme possesses polymerase activity, although false positives were observed. Previous studies by Widyanto *et al*. (2021) and Alhamid *et al* (2023) using the LAMP method also reported false positives. False positives in LAMP assays may result from dimer formation, as LAMP employs multiple high-concentration primers which can lead to primer dimerization (Wang *et al*. 2015; Kim *et al*. 2023). Wang *et al* (2015) confirmed non-specific amplification due to primer dimer formation in LAMP targeting hlyA from Listeria monocytogenes. Cross-contamination can also produce false positives (Kim *et al.* 2023). Strategies to mitigate false positives include reducing primer concentrations to avoid dimer formation and adding substances such as DMSO, betaine, pullulan, and graphene oxide to disrupt secondary structure formation in primers (Widyanto *et al*. 2021; Kim *et al*. 2023).

Acknowledgments

This work was supported by the Indonesian Endowment Fund for Education (LPDP) (LOG-8460/LPDP.3/2024), Ministry of Finance of the Republic of Indonesia and Research granted to RF and AM, and Community Service, and Innovation (PPMI) SITH ITB (27A/IT1.C11/SK-TA/2023) Bandung Institute of Technology (ITB) granted to IT.

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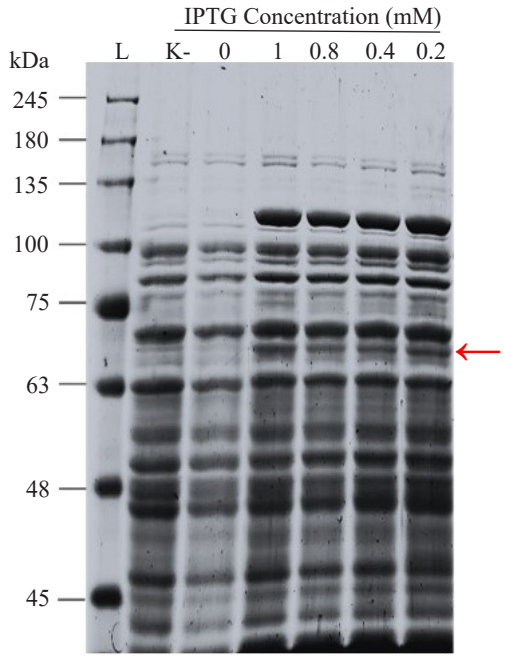
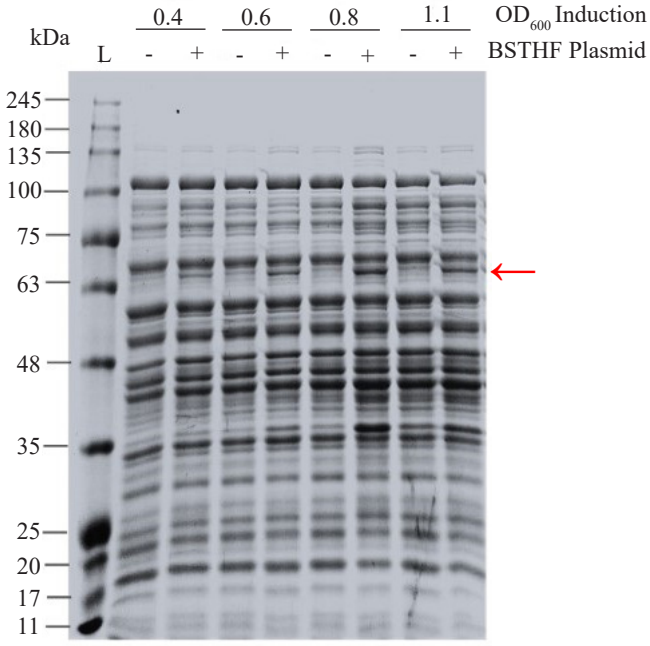
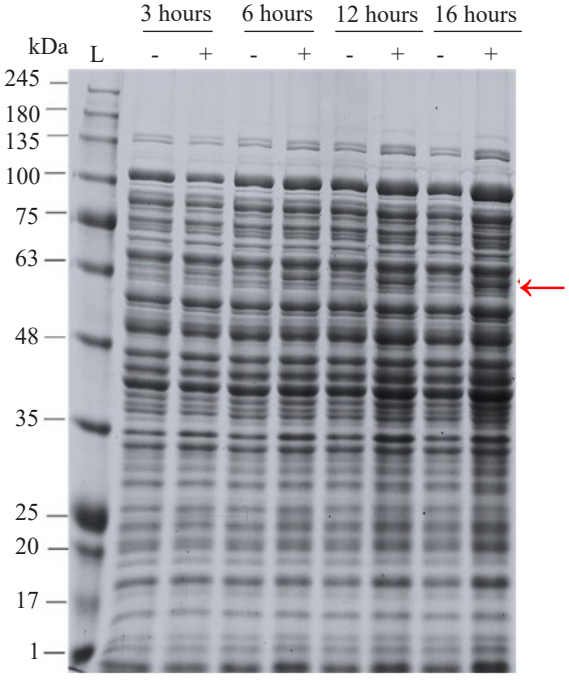
Supplementary Materials



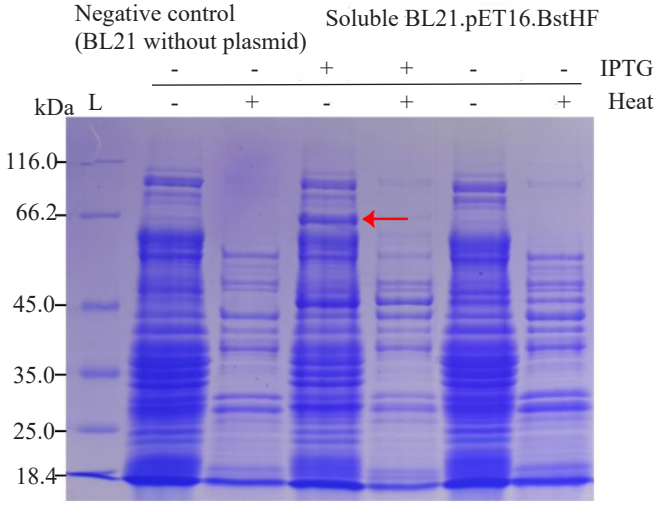
Supplementary Figure 1. SDS-PAGE for visualizing the optimization of IPTG concentration variations; the red arrow indicates the target BSTHF band



Supplementary Figure 2. SDS-PAGE analysis for visualizing the optimization of the IPTG induction point at different OD_{600} values; the red arrow indicates the target BSTHF protein band



Supplementary Figure 3. SDS-PAGE analysis for visualizing the optimization of IPTG induction with varying durations; the red arrow indicates the target BSTHF protein band



Supplementary Figure 4. SDS-PAGE visualization for protein analysis of the negative control (*E. coli* BL21 without plasmid) and *E. coli* BL21.pET16b.BSTHF with and without IPTG induction, and with and without heat treatment. The red arrow indicates the target BSTHF band