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Evaluation of Culture Media and Induction Conditions as a Strategy for Optimising Soluble Human Hexokinase Isoform 2 Expression in *Escherichia coli* BL21 Star™ (DE3)

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ABSTRACT

Hexokinase isoform 2 (HK2) is a rate-limiting enzyme that catalyses the phosphorylation of glucose into glucose-6-phosphate (G6P) in the first step of glycolysis. In cancer cells, HK2 is highly expressed to meet the increased energy demand for rapid proliferation. This overexpression makes HK2 an attractive target for drug development. Nevertheless, getting a pure, soluble recombinant HK2 to study the effects of certain compounds on this enzyme is a challenge due to the large size of the HK2 protein, ~102 kDa. The goal of this study is to optimise recombinant HK2 expression in *Escherichia coli* BL21 Star™ (DE3) through analysing distinct culture media and induction parameters to improve its expression for further production. Three different culture media, Luria-Bertani (LB) Broth Miller, Terrific Broth (TB), and Super Broth (SB), were evaluated in 100 ml and 500 ml. Slow growth was observed in SB media, where it took 3 hours to reach an OD_{600} of 0.4 in a 5% reinoculation culture. Upon induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 hours at 16-18˚C, no detectable HK2 was present in the soluble fraction of SB media. Meanwhile, TB supported rapid growth but yielded a smaller amount of soluble HK2 compared to LB. In a 100 ml culture, LB and TB produced soluble HK2, with TB having a low expression compared to LB. At the 500 ml scale-up, TB yielded insoluble protein, while both soluble and insoluble protein were detected in the LB culture, yet no detectable protein was expressed in either soluble or insoluble fraction of SB media. LB was then chosen as the subsequent medium for testing different IPTG concentrations (0.1 mM, 0.5 mM, and 1.0 mM). Upon investigation, the HK2 band is only visible in 0.1 mM IPTG induction, both in the soluble and inclusion body fractions, while other concentrations produced more insoluble fractions. Compared to prior research in BL21 (DE3), this study demonstrates that BL21 Star™ (DE3) promotes enhanced soluble HK2 expression in LB media upon induction of 0.1 mM IPTG during low-temperature induction (16-18°C, 20 h). This optimisation strategy can facilitate further HK2-related drug discovery and therapeutic research.

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1. Introduction

Hexokinase isoform 2, also known as HK2, is the most crucial enzyme in the human glycolytic pathway that influences cancer metabolism and carcinogenesis. This enzyme catalyzes the first step of glycolysis, where it phosphorylates glucose into glucose-6-phosphate. Besides the importance of HK2 in the glycolytic pathway, the expression of this enzyme or protein has also been shown to be significantly increased in numerous cancer cells, such as lung cancer, laryngeal squamous cell carcinoma, and B-cell lymphoma [1,2]. This enzyme is thus associated with the Warburg effect, in which it accelerates the glycolytic activity of the conversion of glucose to lactate even in the presence of oxygen, a hallmark of cancer cell metabolism [3,4]. This subsequently supports the rapid proliferation of cancer cells. Hence, the downregulation of HK2 could potentially inhibit glycolysis, which will then help in reducing cancer cell growth, making it a promising target for novel drug development [5,6].

In addition to the overexpression of HK2 in cancer and tumour cells, the level of this enzyme increases in the dengue virus (DENV)-infected cells during the post-transcriptional stage, since HK2 mRNA levels stay unchanged [7]. The cyclin-dependent kinases 8 and 19 (CDK8 and CDK19) are two transcriptional regulators that are required for the upregulation of HK2, facilitating viral replication [8]. As the HK2 protein level increases in the infected cells, the glycolytic activity is also observed to be increased, since it provides significant energy and substrates for viral replication [9,10]. Thus, pharmacological inhibition of glycolysis may potentially suppress the HK2 activity and substantially diminish DENV RNA synthesis and infectious cell proliferation [10].

The search for compounds to inhibit HK2 has been ongoing to combat dengue fever. According to previous studies, promising compounds include chitin, 2-deoxyglucose (2DG), and β-N-acetyl-D-galactosamine (GalNAc) [11,12]. To study the inhibition of HK2 using these compounds, producing recombinant HK2 is the first crucial step. This involves cloning the HK2 gene into an expression vector, which is then introduced into a host organism such as *Escherichia coli* [13,14]. *E. coli* is a well-established host for expressing recombinant proteins because of its well-understood genetics, ease of control, and low cost [15,16]. Additionally, the rapid growth rate of *E. coli* allows for quick production and high yields of recombinant protein, making it advantageous for research purposes [16,17]. Apart from that, the versatility and flexibility of this host organism in various expression vectors, such as the pET system, is also one of the reasons for its prominence in the field of research [18,19].

While it is crucial to obtain pure recombinant HK2 protein in the inhibition studies, it is not without its challenges. Obtaining a high-level and soluble expression of recombinant HK2 protein is quite a challenge, as the size of this recombinant protein is quite large, ~102 kDa. Issues like protein solubility and stability require careful optimisation to guarantee that the protein retains its functionality for its use in inhibition studies. To overcome these issues, optimisation of growth media, induction conditions, and expression parameters are necessary.

Three commonly employed culture media, Luria-Bertani (LB) Broth Miller, Terrific Broth (TB), and Super Broth (SB) were evaluated to find the most suitable media that can give a high yield of soluble protein under various induction parameters. Choosing a type of media is crucial since it will significantly influence protein stability and function and enhance the expression level of the protein. Furthermore, the effects of varying induction parameters, including isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (0.1, 0.5, and 1.0 mM) and incubation temperatures (16°C, 25°C, and 37°C) with corresponding induction times, were systematically assessed to identify conditions most conducive to soluble protein expression.

Previous research on HK2 has primarily focused on its enzymatic activity and therapeutic potential, while systematic optimisation of its recombinant expression in *E. coli* has been largely overlooked. This gap limits the ability to obtain sufficient quantities of soluble HK2 for biochemical, structural, and inhibitor studies. Addressing this limitation is essential to accelerate HK2-targeted drug discovery. Thus, the significance of this study lies in its potential to develop a reproducible and scalable optimisation for HK2 production, which would facilitate structural and functional studies that may aid in novel drug discovery. Therefore, the primary goals of this study are: (1) to compare different culture media for HK2 expression in *E. coli* BL21 Star™ (DE3), (2) to assess the effects of IPTG concentration, induction temperature, and induction duration on HK2 solubility, and (3) to determine the best conditions for maximizing soluble HK2 expression for future large-scale applications.

2. Methodology

2.1 Bacterial Transformation

Bacterial transformation was performed using chemically competent *E. coli* BL21 Star™ (DE3) cells (Thermo Fisher Scientific). This step ensured the incorporation of the foreign DNA plasmid, specifically the pET28b-HK2 plasmid, into the bacterial cells. The transformation was carried out using a heat shock method, which involved exposing the cells to a sudden temperature shift to facilitate DNA uptake. Briefly, 1 µL of the plasmid (50 ng/µL) was added to 100 µL of competent cells, and the mixture was incubated on ice for 30 minutes. The cells then underwent heat shock at 42 °C for 1 minute and were immediately returned to ice and incubated for an additional 2 minutes. This temperature shift created a transient thermal imbalance that briefly permeabilized the cell membrane, allowing plasmid DNA to enter the cells [20]. Following heat shock, 900 µL of room-temperature LB broth was added (SOC media could alternatively be used), and the mixture was incubated at 37 °C with shaking at 400 to 600 rpm for 2 to 3 hours. After incubation, the mixture was centrifuged at maximum speed for 1 minute, 900 µL of the supernatant was discarded, and the remaining 100 µL containing the transformants was plated on LB agar supplemented with 50 µg/mL kanamycin. Plates were incubated overnight at 37 °C. The overall procedure of bacterial transformation is illustrated in Figure 1.

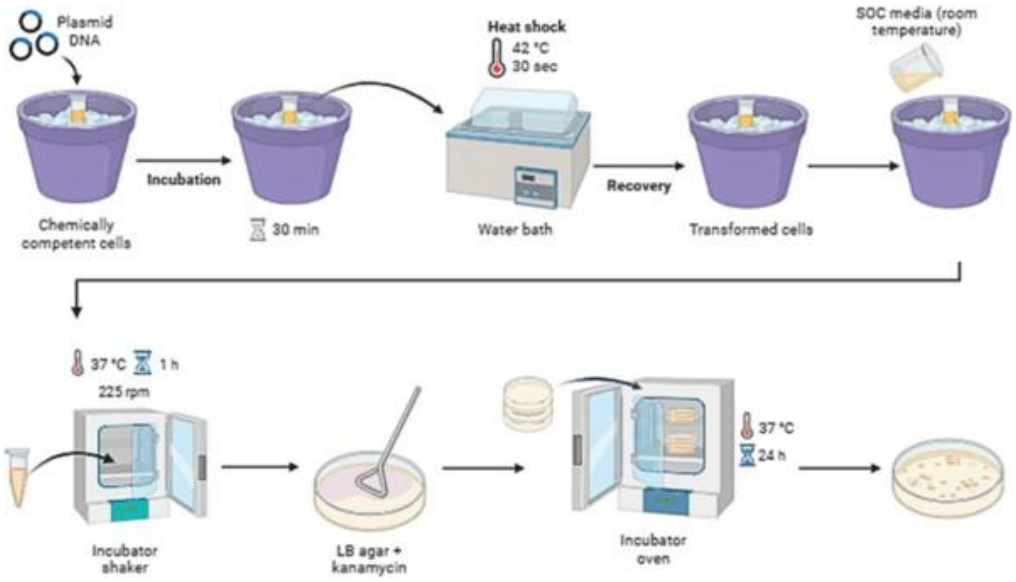


Fig. 1. Bacterial transformation overall procedure

2.2 Colony Polymerase Chain Reaction (PCR)

Colony PCR was performed to validate the presence of the HK2 insert in the transformants by selecting a single colony from the bacterial transformation. The procedure was carried out using the Thermo Scientific Phusion™ High-Fidelity PCR Kit. The components for a 20 µL reaction are shown in Table 1. The extracted pET28b-HK2 plasmid was used as the positive control.

Table 1
Colony PCR components

Component	Volume (µl)
Distilled water (dH_2O)	To 20 µl
5X Phusion HF Buffer	4
10 mM dNTPs	0.4
Forward primer	1
Reverse primer	1
DMSO (optional)	0.6
Phusion DNA Polymerase	0.2
DNA template	1
FINAL TOTAL	**20 µl**

The forward and reverse primers used in this study were the T7 promoter and terminator primers, which are universal primers. Table 2 shows the sequences of these oligonucleotide primers.

Table 2
The sequence of primers used in the PCR

Primer	Sequence
T7 promoter (forward)	5'-TAA TAC GAC TCA CTA TAG G-3'
T7 terminator (reverse)	5'-CTA GTT ATT GCT AGT CGT GAT GGC GCA ATG AAC G-3'

The PCR began with an initial denaturation, followed by 29 cycles of denaturation, annealing, and extension, with a final extension step. The PCR amplification profile is shown in Table 3. Following colony PCR, 0.8% agarose gel electrophoresis was performed to visualize the results under UV light. The agarose gel was prepared by dissolving 0.25 g of biotechnology-grade agarose in 40 mL of 1X TAE buffer by heating until fully dissolved. Two microliters of SYBR™ Safe DNA Gel Stain were added to enable DNA visualization. Once the gel had solidified, the PCR products were loaded into the wells, and electrophoresis was conducted at 80 V for 60 minutes. The results were subsequently visualized under UV light.

Table 3
Colony PCR profile

Step	Temperature (˚C)	Duration
1. Initial denaturation	98	45 sec
2. Denaturation	98	10 sec
3. Annealing	50	30 sec
4. Extension	72	1 min 30 sec
Go to step 2 for 29 cycles		
5. Final extension	72	5 min
6. Keep	12	∞

2.3 Restriction Enzyme Digestion Analysis

Restriction enzyme digestion is an important molecular biology technique that uses restriction endonucleases to cleave DNA at specific sites, allowing analysis of DNA fragments containing the insert [21]. Combining RE analysis with PCR is a widely used method for identifying transgenic insertion sites [21]. In this study, the HK2 insert was ligated into the pET28b vector between the BamHI and HindIII restriction sites. RE analysis was performed using a restriction enzyme kit from Promega. Both single and double digestions were carried out. The reactions were prepared by mixing distilled water, 10X restriction enzyme buffer, 10 µg/µL acetylated BSA, 1 µg/µL plasmid DNA from the transformants, and the appropriate restriction enzymes (BamHI and HindIII) according to the specific volumes listed in Table 4. The mixtures were incubated for 2 hours and subsequently analysed by 0.8% agarose gel electrophoresis under UV illumination.

Table 4
Restriction enzyme digestion components

Component	Double digestion volume (µl)	Single (BamHI) digestion volume (µl)	Single (BamHI) digestion volume (µl)
Distilled water (dH_2O)	To 20 µl	To 20 µl	To 20 µl
Restriction enzyme 10X buffer	2	2	2
Acetylated BSA, 10 µg/µl	0.4	0.4	0.4
BamHI (10 U/µl)	1	1	-
HindIII (10 U/µl)	1	-	1
Plasmid DNA	1 µg/µl	1 µg/µl	1 µg/µl
FINAL TOTAL	**20 µl**	**20 µl**	**20 µl**

2.4 Protein Expression

Once the transformants were confirmed to contain the HK2 insert, protein expression was carried out. The primary objective was to obtain soluble protein for subsequent purification and inhibition studies in the development of novel anti-cancer drugs.

2.4.1 Protein expression optimisation strategies using one-factor-at-a-time (OFAT) experimental design

The optimisation strategies of HK2 expression involved a series of experiments employing one-factor-at-a-time (OFAT). This experimental design allowed a single variable to be varied while keeping all other variables constant. In this study, three stages of OFAT were employed to determine the most suitable parameters for scale-up. The variables investigated included the type of growth media (LB, TB, and SB), IPTG concentration (0.1, 0.5, and 1.0 mM), temperature (16, 25, and 37 °C), and induction time according to temperature (20 hours for 16 °C, 6 hours for 25 °C, and 4 hours for 37 °C).

In the first stage, different types of media were tested to identify which supported optimal transformant growth and soluble HK2 production, while keeping the temperature, IPTG concentration, and induction time constant. The OFAT design for the first stage is presented in Table 5.

Table 5
First stage of OFAT for protein expression, varying the type of media

Manipulated Variable	Constant Variables		
Type of media	Temperature (˚C)	IPTG concentration (Mm)	Induction time (hours)
LB TB SB	16	0.1	20

Once the most suitable media was identified, the second stage of OFAT was conducted by varying the IPTG concentrations. Table 6 presents the manipulated variable alongside those that were kept constant.

Table 6
Second stage of OFAT for protein expression, varying the IPTG concentration

Manipulated Variable	Constant Variables		
IPTG concentration (mM)	Type of media	Temperature (˚C)	Induction time (hours)
0.1 0.5 1.0	LB/TB/SB	16	20

The final stage of OFAT for protein expression involved varying the induction temperature along with the corresponding induction time. The relationship between induction temperature and induction time reflects a complex interaction of thermodynamic and kinetic parameters. The induction time was adjusted according to temperature due to differences in energy barriers and reaction kinetics, where higher temperatures typically provide additional energy to overcome these barriers, thereby shortening the induction time [22,23]. Accordingly, the lowest temperature tested, 16 °C, underwent a 20 hours induction period, while 25 °C and 37 °C underwent 6 and 4 hours induction periods, respectively, as shown in Table 7.

Table 7
The third stage of OFAT for protein expression, varying the temperature and induction time

Manipulated Variable	Constant Variables		
Temperature (˚C)	Type of media	IPTG concentration (mM)	Induction time (hours)
16 25 37	LB/TB/SB	0.1/0.5/1.0	20 6 4

2.4.2 HK2 protein expression in E. coli BL21 Star™ (DE3)

The first stage of protein expression began with a 10 mL starter culture prepared in the media indicated in Table 5, containing 30 µg/mL kanamycin. After overnight incubation at 37 °C with shaking at 250 rpm, the starter culture was subcultured into fresh media and incubated at 37 °C with shaking at 150 rpm until the OD_{600} reached 0.6 to 0.8. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for 20 hours. The following day, the culture was harvested by centrifugation at 8,000 rpm and 4 °C for 15 minutes, and the supernatant was discarded. The pellet was resuspended in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 10% glycerol [optional], pH 8.0) and homogenized for 10 minutes before being physically lysed using a sonicator for 3.5 minutes with 10 seconds pulses on and 30 seconds pulses off at 35% amplitude. The lysate was then centrifuged at 10,000 rpm for 15 to 20 minutes at 4 °C. The resulting

supernatant, containing the expressed protein, was analysed by 8% SDS-PAGE, while the pellet was saved for inclusion body (IB) analysis. Based on these results, the experiment was repeated according to the parameters outlined in Tables 6 and 7.

2.5 Dot Blot Analysis

Dot blotting is a simple technique used to detect and quantify biomolecules, such as proteins, nucleic acids, and antibodies. This method involves the direct application of small amounts of samples onto a membrane (usually nitrocellulose or polyvinylidene difluoride (PVDF)) to detect the presence of target biomolecules. In this study, a nitrocellulose membrane was used to capture the biomolecules, which were subsequently visualized using chemiluminescence.

A total of 30 µL of samples was directly spotted onto the membrane, with 10 µL per spot, alternating with air-drying steps between each application to enhance signal and concentration. The membrane was then blocked with 5% skimmed milk in 1X PBST and incubated for 1 hour at room temperature to prevent non-specific binding. Following blocking, the membrane was washed four times with 1X PBST for 5 minutes each. The membrane was then incubated in 5% skimmed milk in 1X PBST containing 1 µL of His-tag antibody (horseradish peroxidase [HRP] conjugate, 1:25,000) on a belly dancer for 1 hour at room temperature. Because the antibody was HRP-conjugated, secondary antibody incubation was not required. The membrane was then washed four times with 1X PBST for 5 minutes each before proceeding to detection using chemiluminescence.

2.5 Western Blot Analysis

Western blot analysis shares a key similarity with the dot blot assay, as both are immunoassay techniques used for protein detection. Unlike dot blotting, western blotting is more complex and offers high specificity by separating proteins based on their molecular weight through electrophoresis before transferring them onto a membrane [24–26].

Western blotting began with protein separation by SDS-PAGE. The separated proteins were then transferred onto a nitrocellulose membrane using transfer buffer (25 mM Tris base, 192 mM glycine, 20% [v/v] methanol, dH_2O). Subsequent steps were similar to dot blotting, starting with blocking, followed by antibody incubation, and visualization using chemiluminescence. Non-specific binding sites were blocked with 5% skimmed milk in 1X PBST for 1 hour on a rocker and then washed four times with 1X PBST for 5 minutes each. The membrane was incubated with 6x His-tag antibody (HRP conjugate) in 5% skimmed milk at a 1:25,000 dilution for 1 hour at room temperature. Protein detection was achieved using chemiluminescence.

3. Results

3.1 Bacterial Transformation

The results of bacterial transformation, involving the incorporation of the pET28b-HK2 plasmid into *E. coli* BL21 Star™ (DE3), were confirmed by the appearance of single colonies on LB agar containing kanamycin. Kanamycin served as a selective agent, allowing only successfully transformed cells carrying the plasmid, which includes an antibiotic resistance gene (pET28b), to grow [28]. Following heat shock at 42 °C, multiple single colonies were obtained (Figure 2), indicating successful transformation of the competent cells with the recombinant HK2 plasmid. These results demonstrate the effectiveness of the employed methods for subsequent studies.



Fig. 2. A few single colonies on LB agar plates indicate successful transformation

3.2 Colony Polymerase Chain Reaction

Single colonies on transformation plates can sometimes represent false positives due to factors such as mutations or contamination [29,30]. To identify positive transformants, colony PCR was performed. Using vector-specific T7 promoter and T7 terminator primers, the expected amplicon size corresponded to the length of the insert plus the 5' vector segment (from the T7 promoter primer to the 5' cloning site) and the 3' vector segment (from the 3' cloning site to the T7 terminator primer), as described in Eq. (1).

$$Expected\ size = Insert\ length + 4Promoter - to - 5^{'}\ site: + (3^{'}\ site - to - Terminator) \quad (1)$$

Given that the HK2 insert is approximately 2,754 bp, the vector segment from the 5' T7 promoter to the BamHI site is 146 bp, and the vector segment from the HindIII site to the 3' T7 terminator is 183 bp, the expected amplicon size was 3,083 bp. As shown in Figure 3, the transformants produced amplicons of the expected size, confirming the presence of the target gene. The confirmed transformants were then subjected to further analyses to validate the insert. This approach ensured that downstream experiments were conducted using clones carrying the correct recombinant plasmid.

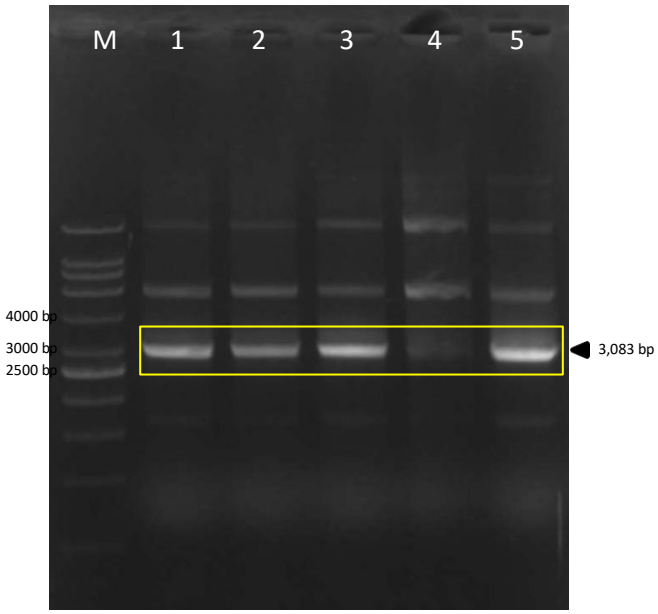


Fig. 3. The result from colony PCR using T7 promoter and terminator primers. The expected band is as highlighted in the yellow box, where, M: 1 kB DNA marker, 1 and 2: first transformant, 3 and 4: second transformant, and 5: positive control (extracted pET28b-HK2 plasmid)

3.3 Restriction Enzyme (RE) Digestion

To further confirm the presence of the insert, restriction enzyme (RE) analysis was performed on plasmid DNA extracted from positive colonies using BamHI and HindIII, which flank the insert. The digested plasmid was separated on a 0.8% agarose gel, revealing a single band of 8,122 bp in the single digestion, corresponding to the size of the recombinant plasmid (pET28b-HK2), and two bands in the double digestion: one at 5,368 bp corresponding to the pET28b vector backbone and another at 2,754 bp corresponding to the HK2 insert. As shown in Figure 4, the observed fragments confirmed that the insert was correctly ligated into the pET28b vector and that the plasmid construct was properly assembled. This analysis, together with colony PCR, validated the recombinant plasmid for subsequent protein expression experiments.

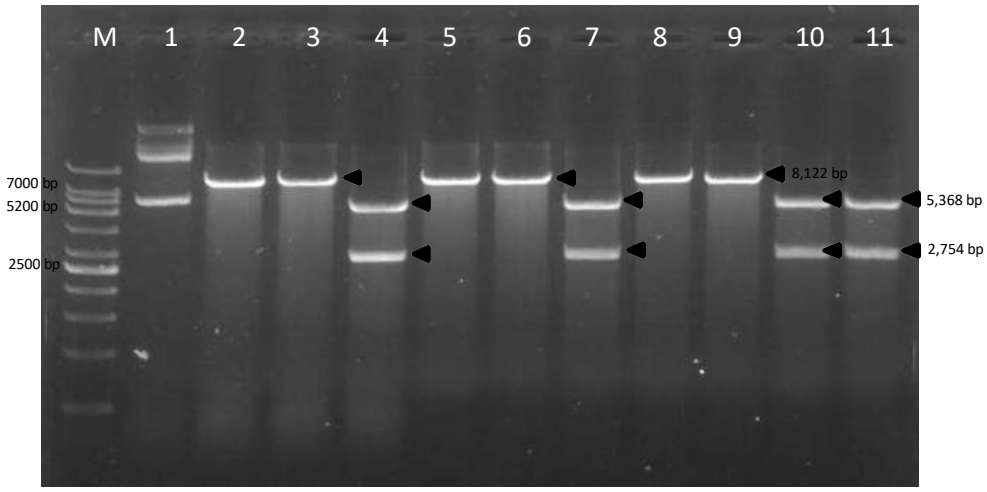


Fig. 4. 0.8% of agarose gel electrophoresis for RE digestion, where; M: 1 kB DNA marker, 1: uncut plasmid, 2: positive control (BamHI single digestion), 3: positive control (HindIII single digestion), 4: positive control (double digestion), 5: transformant 1 (BamHI single digestion), 6: transformant 1 (HindIII single digestion), 7: transformant 1 (double digestion), 8: transformant 2 (BamHI single digestion), 9: transformant 2 (HindIII single digestion), 10: transformant 2 (double digestion), and 11: transformant 3 (double digestion)

3.4 Recombinant HK2 Protein Expression

3.4.1 Stage 1: 100 mL expression in LB, TB, and SB media (0.1 mM IPTG, 16°C, 20h)

During the first stage of protein expression, three media, which are LB, TB, and SB broth, were used to determine the most suitable medium for optimizing expression conditions. Small-scale cultures (100 mL) were evaluated by monitoring the OD_{600} of the starter cultures after 1% subculture (5 mL into 500 mL). As shown in Table 8, TB supported the fastest growth, reaching an OD_{600} of approximately 0.6 to 0.8 within 1 hour, followed by LB, which reached a similar OD_{600} after approximately 1 hour and 15 minutes. In contrast, cultures in SB media grew more slowly, taking around 3 hours to reach an OD_{600} of approximately 0.4. These findings are consistent with the nutrient richness of TB, which contains high concentrations of peptone, yeast extract, and glycerol, providing a strong carbon source [31]. Although SB is also nutrient-rich, its performance may vary depending on bacterial strain or formulation, possibly due to differences in nutrient composition or the presence of growth stimulators or inhibitors [32,33].

Table 8
OD_{600} reading of 3 different culture media

Media/Time	1 hour	1 hour 15 minutes	3 hours
LB	0.449	0.620	-
TB	0.688	-	-
SB	0.204	0.254	0.409

SDS-PAGE analysis of the first-stage expression revealed HK2 bands near the expected molecular weight (~102 kDa) in the soluble fractions of induced LB and TB cultures, while SB also produced a detectable but weaker band (Figure 5). In the insoluble or inclusion body (IB) fractions (Figure 7), all three media displayed strong expression at the same molecular weight, indicating that a significant portion of the protein was directed into inclusion bodies.

Dot blot analysis of the first stage revealed signals in both induced and uninduced samples for soluble (Figure 9) and insoluble fractions (Figure 10) using an anti-His tag antibody conjugated to HRP. This indicated that detection was not limited to IPTG-induced expression of the target HK2 protein, as the His tag is located at the N-terminus of the construct; therefore, the antibody may have detected basal expression in the uninduced samples. Because dot blotting cannot distinguish proteins by molecular size, the observed signals could not differentiate full-length HK2 from smaller His-tagged polypeptides or non-specific His-tagged fragments. Consequently, Western blot analysis was performed to confirm the size and specificity of the expressed HK2 protein.

Western blot analysis of the first-stage 100 mL cultures (Figure 6) revealed a distinct band at ~102 kDa in the induced samples of LB and TB media, whereas no band was detected in the induced sample of SB media (Figure 8). The absence of bands in the uninduced controls confirmed that expression was IPTG-dependent, indicating that the dot blot signals in uninduced samples likely represented basal expression. In the insoluble fraction, induced LB and TB cultures produced positive signals, although the bands appeared smeared, suggesting partial aggregation or degradation. Overall, these data indicate that LB and TB support soluble HK2 production, whereas SB is less effective, and that inclusion body formation occurs in all media. Based on these findings, LB and TB were selected for scale-up, while SB was excluded due to slower growth and lower protein expression.

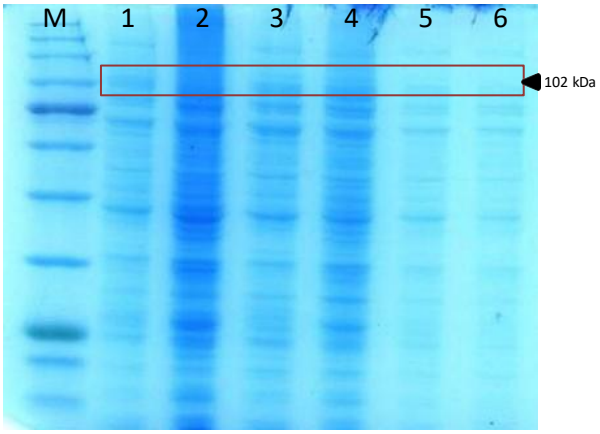


Fig. 5. SDS-PAGE of the soluble fraction, where M: protein marker 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, and 6: expressed protein in SB media (uninduced). The expressed protein bands are highlighted in the red box

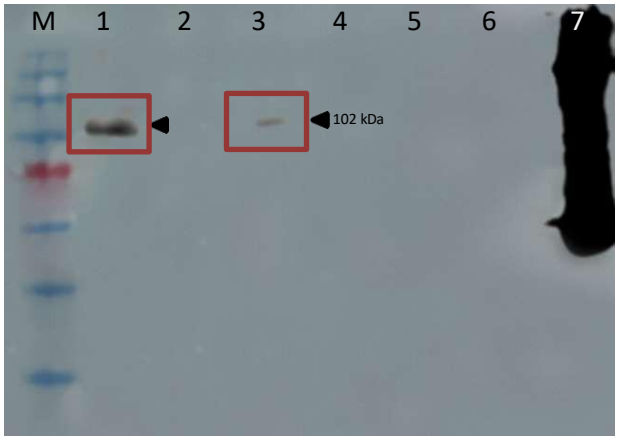


Fig. 6. Western blot result of the soluble fraction, where; M: Western marker (PM2700), 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, 6: expressed protein in SB media (uninduced), and 7: positive control (Zn Protease protein). The expressed protein bands are highlighted in the red box

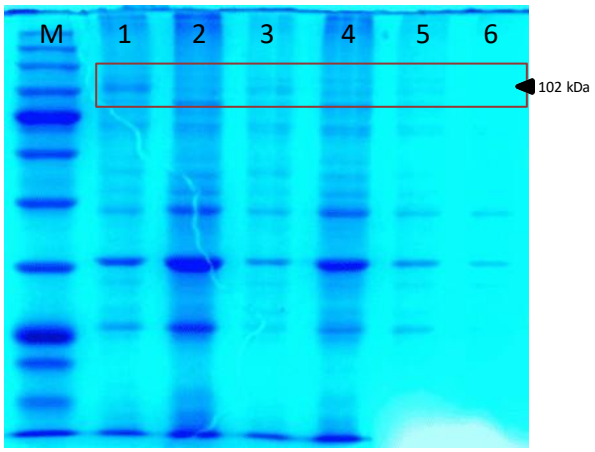


Fig. 7. SDS-PAGE of the insoluble fraction, where M: protein marker 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, and 6: expressed protein in SB media (uninduced). The expressed protein bands are highlighted in the red box

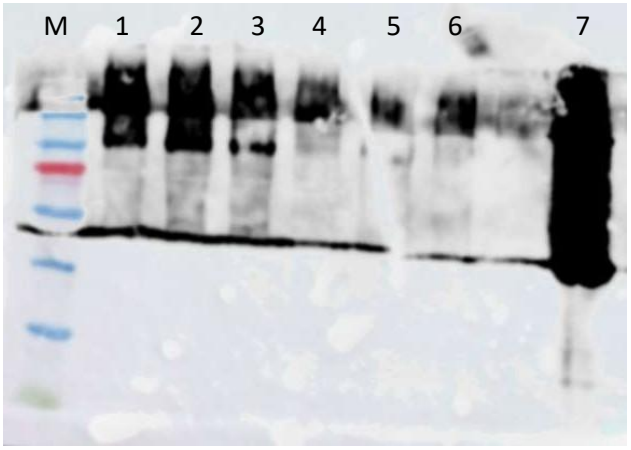


Fig. 8. Western blot result of the insoluble fraction, where; M: Western marker (PM2700), 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, 6: expressed protein in SB media (uninduced), and 7: positive control (Zn Protease protein)

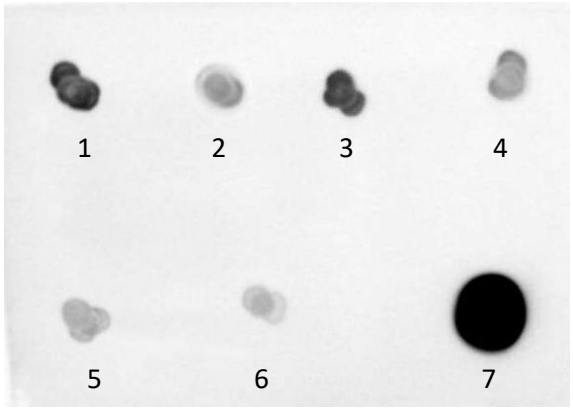


Fig. 9. Dot blot result of the soluble fraction, where; 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, 6: expressed protein in SB media (uninduced), and 7: positive control (Zn Protease protein)

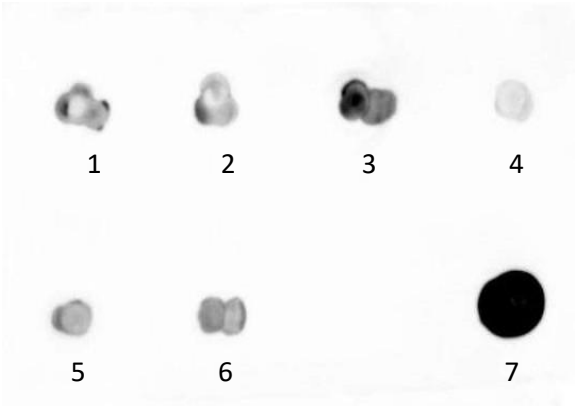


Fig. 10. Dot blot result of the insoluble fraction, where; 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: expressed protein in SB media, 6: expressed protein in SB media (uninduced), and 7: positive control (Zn Protease protein)

3.4.2 Stage 2: 500 mL expression in LB and TB media (0.1 mM IPTG, 16°C, 20h)

In the second stage, SDS-PAGE revealed a clear ~102 kDa band in the soluble fraction of LB media only (Figure 11), while strong bands were observed in the insoluble fractions of both LB and TB media (Figure 13). These results were corroborated by Western blotting, which showed a distinct signal in the soluble fraction of LB-induced samples, whereas no soluble signal was detected in TB cultures (Figure 12). In the insoluble fractions (Figure 14), both LB and TB displayed strong signals, indicating

high levels of expression but predominantly misfolded protein. The discrepancy between SDS-PAGE and Western blot results for TB media suggests that most of the protein was insoluble or improperly folded, limiting antibody detection in the soluble fraction. In contrast, LB media supported soluble HK2 expression, detectable by both SDS-PAGE and Western blot. Based on these data, LB media was selected for further large-scale expression, while TB media was excluded.

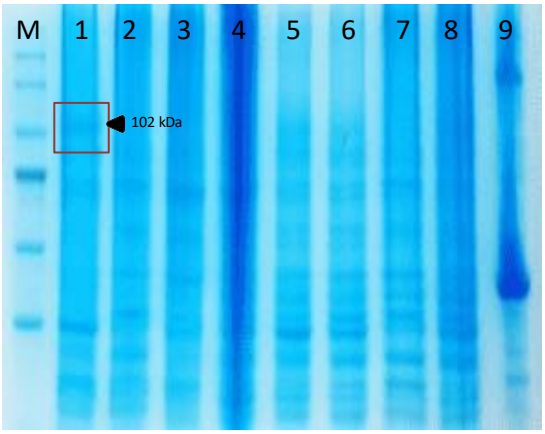


Fig. 11. SDS-PAGE result of the soluble fraction, where; M: protein marker, 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: empty host (control) expressed in LB media (induced), 6: empty host (control) expressed in LB media (uninduced), 7: empty host (control) expressed in TB media (induced), 8: empty host (control) expressed in TB media (uninduced), and 9: positive control (Zn Protease protein). The expressed protein bands are highlighted in the red box

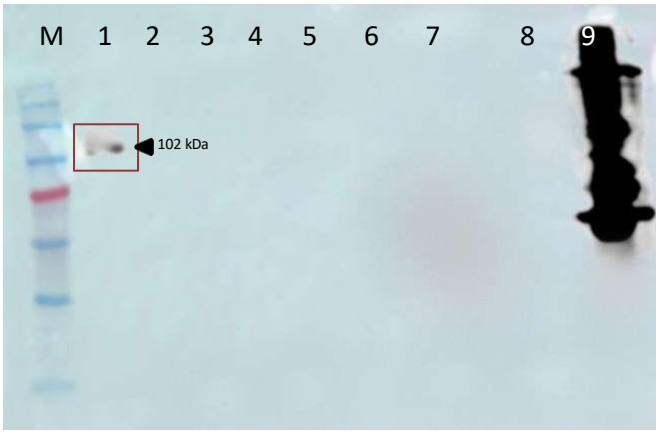


Fig. 12. Western blot result of the soluble fraction, where; M: Western marker (PM2700), 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: empty host (control) expressed in LB media (induced), 6: empty host (control) expressed in LB media (uninduced), 7: empty host (control) expressed in TB media (induced), 8: empty host (control) expressed in TB media (uninduced), and 9: positive control (Zn Protease protein). The expressed protein bands are highlighted in the red box

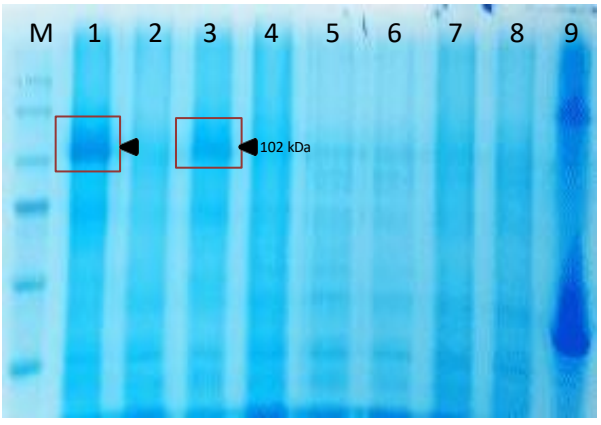


Fig. 13. SDS-PAGE result of the insoluble fraction, where; M: protein marker, 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: empty host (control) expressed in LB media



Fig. 14. Western blot result of the insoluble fraction, where; M: Western marker (PM2700), 1: expressed protein in LB media, 2: expressed protein in LB media (uninduced), 3: expressed protein in TB media, 4: expressed protein in TB media (uninduced), 5: empty host (control) expressed in LB media (induced), 6:

(induced), 6: empty host (control) expressed in LB media (uninduced), 7: empty host (control) expressed in TB media (induced), 8: empty host (control) expressed in TB media (uninduced), and 9: positive control (Zn Protease protein). The expressed protein bands are highlighted in the red box

empty host (control) expressed in LB media (uninduced), 7: empty host (control) expressed in TB media (induced), 8: empty host (control) expressed in TB media (uninduced), and 9: positive control (Zn Protease protein)

3.4.3 Stage 3: 2 L expression in LB media, varying IPTG concentration, induction temperature, and induction time

Large-scale expression was optimised by varying IPTG concentrations and induction temperatures. SDS-PAGE analysis of the soluble fractions (Figure 15) revealed the clearest band at ~102 kDa under conditions of 0.1 mM IPTG and 16 °C with 20 hours of induction. The weakest signal was observed at 37 °C with 0.1 mM IPTG for 4 hours, where no distinct band was detectable. Strong bands were consistently observed in the insoluble fractions under all conditions (Figure 17), indicating the natural tendency of the protein to aggregate.

These results were further validated by Western blotting, with soluble fractions shown in Figure 16 and insoluble fractions in Figure 18. 50 µg of HK2 protein were detected in all soluble fraction conditions, although the signals appeared smeared except for the 37 °C, 4 hours condition. This smearing likely resulted from inclusion body aggregation under metabolic stress at high temperature and short induction time, suggesting that these conditions produced mostly misfolded, insoluble HK2 with minimal soluble protein [34,35]. As observed in the 500 mL scale, bands in the insoluble fractions were more prominent, confirming protein aggregation into inclusion bodies. Nonetheless, the presence of the 102 kDa band in soluble fractions under other conditions confirmed that the recombinant protein was functional and capable of generating soluble HK2. Collectively, these data indicate that LB media supports optimal soluble HK2 expression at 0.1 mM IPTG with 20 hours of induction at 16 °C.

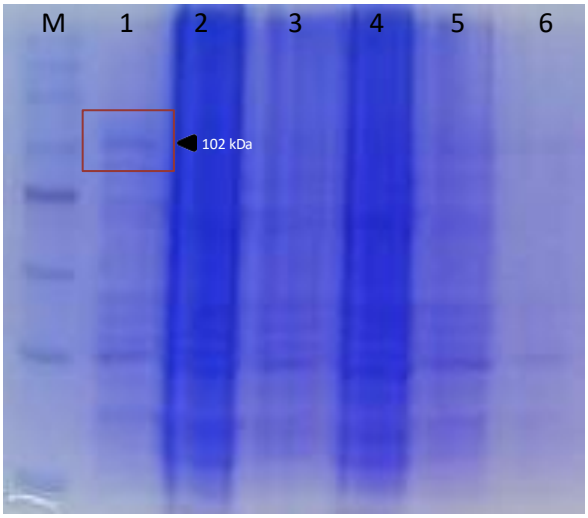


Fig. 15. SDS-PAGE result of the soluble fraction for protein expression in LB media, varying different IPTG concentration and different temperature, where, M: Western marker (PM2700), 1: 0.1 mM IPTG, 16˚C, 20 hours; 2: 0.5 mM IPTG, 16˚C, 20 hours; 3: 1.0 mM IPTG, 16˚C, 20 hours; 4: 0.1 mM

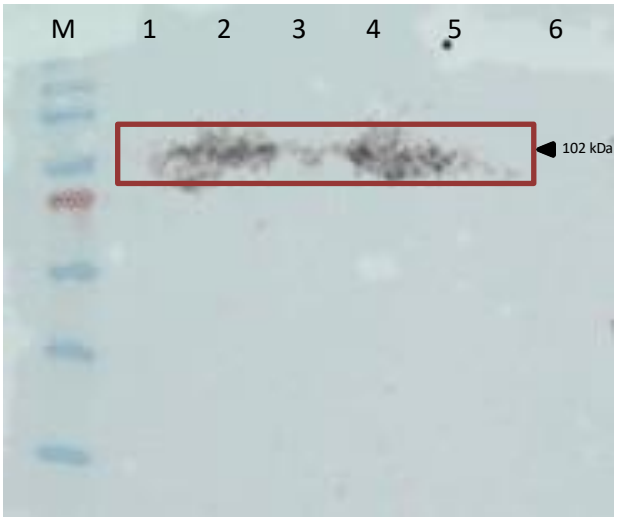


Fig. 16. Western blot result of the soluble fraction for protein expression in LB media, varying different IPTG concentration and different temperature, where, M: Western marker (PM2700), 1: 0.1 mM IPTG, 16˚C, 20 hours; 2: 0.5 mM IPTG, 16˚C, 20 hours; 3: 1.0 mM IPTG, 16˚C, 20 hours; 4: 0.1 mM

IPTG, 16˚C, 20 hour; 5: 0.1 mM IPTG, 25˚C, 6 hours; 6: 0.1 Mm IPTG, 37˚C, 4 hours. The expressed protein (~102 kDa) bands are highlighted in the red box

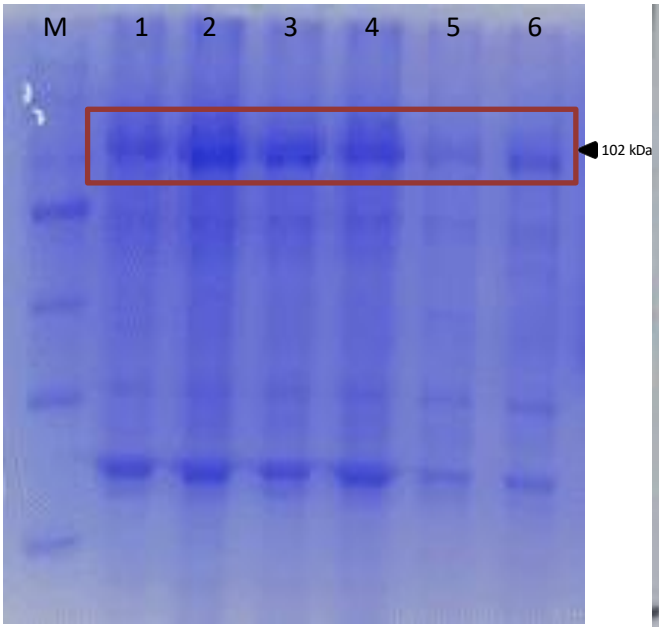


Fig. 17. Western blot result of the insoluble fraction for protein expression in LB media, varying different IPTG concentration and different temperature, where, M: Western marker (PM2700), 1: 0.1 mM IPTG, 16˚C, 20 hours; 2: 0.5 mM IPTG, 16˚C, 20 hours; 3: 1.0 mM IPTG, 16˚C, 20 hours; 4: 0.1 mM IPTG, 16˚C, 20 hour; 5: 0.1 mM IPTG, 25˚C, 6 hours; 6: 0.1 Mm IPTG, 37˚C, 4 hours; 7: positive control (Zn Protease protein). The expressed insoluble protein (~102 kDa) bands are highlighted in the red box

IPTG, 16˚C, 20 hour; 5: 0.1 mM IPTG, 25˚C, 6 hours; 6: 0.1 Mm IPTG, 37˚C, 4 hours. The expressed protein (~102 kDa) bands are highlighted in the red box

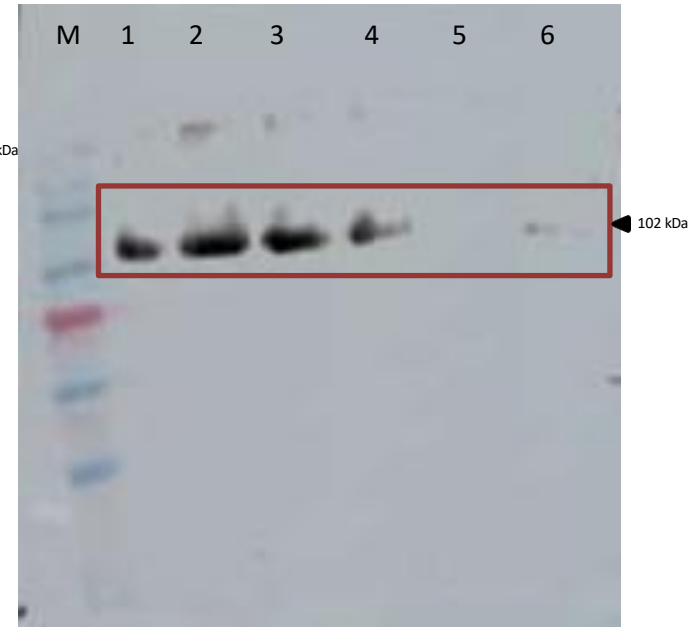


Fig. 18. Western blot result of the insoluble fraction for protein expression in LB media, varying different IPTG concentration and different temperature, where, M: Western marker (PM2700), 1: 0.1 mM IPTG, 16˚C, 20 hours; 2: 0.5 mM IPTG, 16˚C, 20 hours; 3: 1.0 mM IPTG, 16˚C, 20 hours; 4: 0.1 mM IPTG, 16˚C, 20 hours; 5: 0.1 mM IPTG, 25˚C, 6 hours; 6: 0.1 mM IPTG, 37˚C, 4 hours; 7: positive control (Zn Protease protein). The expressed insoluble protein (~102 kDa) bands are highlighted in the red box

4. Conclusions

To conclude, the research effectively expressed and evaluated the recombinant HK2 protein in *E. coli* BL21 Star™ (DE3) under various growth media and expression conditions. Among the investigated media, LB broth showed the most constant soluble expression of HK2, as validated by SDS-PAGE, dot blot, and Western blot analyses. Although inclusion body formation was detected in all conditions, soluble HK2 was most commonly obtained in LB-grown cultures, indicating its feasibility for large-scale production. These outcomes directly address the purpose of this study by determining the best conditions for soluble HK2 expression, which is critical for subsequent purification and functional studies. Considering that HK2 is an essential glycolysis enzyme and has been linked to a variety of metabolic and pathological processes, the development of these optimal expression systems lays the groundwork for future structural, biochemical, and therapeutic research.

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