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THE EFFECT OF CONCENTRATION AND TIME VARIATION OF THE LACTOSE AND SKIM MILK ON THE EXPRESSION OF RECOMBINANT PROTEIN GST-A276R AFRICAN SWINE FEVER VIRUS

Pengaruh Variasi Konsentrasi dan Waktu Induksi Laktosa dan Susu Skim terhadap Ekspresi Protein Rekombinan GST-A276R Virus *African Swine Fever*

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Abstract

Recombinant protein expression in *Escherichia coli* (*E. coli*) commonly utilizes isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer. However, IPTG causes toxicity, metabolic instability, and high cost. Lactose and skim milk provide safer and more economical alternatives and can induce the lac promoter in a more controlled manner. This study aimed to evaluate the effectiveness of lactose and skim milk as alternative inductors for expressing the GST-A276R fusion protein, a candidate subunit vaccine for African Swine Fever (ASF). The A276R gene from ASF virus was inserted into the pGEX-2TK plasmid to produce this fusion protein. Different inducer concentrations (1%, 2.5%, and 5%) and induction times (5, 24, and 48 hours) were evaluated in *E. coli* BL21 cultures using a randomized block design. Intensity of protein expression was analyzed by the dot blot method using anti-GST antibodies, and signal intensity was measured with ImageJ software. The results showed that the type of inducer had a significant effect on the expression level of GST-A276R protein ($p < 0.05$). The combination of 2.5% skim milk and 48 hours of skim milk induced expression was the most optimal setup for GST-A276R protein expression. In addition to inducer type, inducer concentration and induction time also had significant effects on protein expression ($p < 0.05$). Based on these findings, it is concluded that skim milk serves as an effective alternative inducer for GST-A276R protein production. Further analysis is required to test the purity and biological activity of the GST-A276R protein expressed under these optimal conditions.

Keywords: GST-A276R, IPTG, lactose, protein expression, skim milk

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Abstrak

Ekspresi protein rekombinan pada *Escherichia coli* (*E. coli*) banyak menggunakan (IPTG), namun penggunaannya menyebabkan toksisitas, ketidakstabilan metabolik, dan biaya yang tinggi. Laktosa dan susu skim menawarkan alternatif yang lebih aman dan ekonomis serta mampu menginduksi promoter *lac* secara lebih terkontrol. Penelitian ini bertujuan mengevaluasi efektivitas laktosa dan susu skim sebagai alternatif induktor dalam mengekspresikan protein fusi GST-A276R, kandidat vaksin subunit *African Swine Fever* (ASF). Gen A276R dari virus ASF telah disisipkan ke dalam plasmid pGEX-2TK untuk tujuan tersebut. Berbagai konsentrasi induktor (1%, 2,5%, dan 5%) dan waktu induksi (5, 24, dan 48 jam) diuji pada kultur *E. coli* BL21 menggunakan rancangan acak kelompok. Intensitas ekspresi protein dianalisis dengan metode *dot blot* menggunakan antibodi anti-GST, dan intensitas sinyal diukur menggunakan perangkat lunak *ImageJ*. Hasil penelitian menunjukkan bahwa jenis induktor berpengaruh sangat nyata terhadap tingkat ekspresi protein GST-A276R ($p < 0,05$). Kombinasi perlakuan susu skim 2,5% dengan waktu induksi 48 jam merupakan kondisi paling optimal untuk mengekspresikan protein GST-A276R. Selain jenis induktor, konsentrasi dan waktu induksi juga berpengaruh nyata terhadap ekspresi protein ($p < 0,05$). Berdasarkan temuan tersebut, dapat disimpulkan bahwa susu skim berpotensi sebagai induktor alternatif yang efektif untuk produksi protein GST-A276R. Analisis lanjutan diperlukan untuk menguji kemurnian dan aktivitas biologis protein GST-A276R hasil ekspresi dengan kondisi optimal tersebut.

Kata kunci: Ekspresi protein, GST-A276R, IPTG, laktosa, susu skim

INTRODUCTION

Recombinant protein production is an important technology with broad applications across fields ranging from healthcare to agriculture. In medicine, recombinant proteins are used to produce insulin, vaccines, and therapeutic antibodies, while in animal husbandry and agriculture this technology supports the development of veterinary vaccines, nutritional enzymes, and transgenic crops (Bustamante-Córdova *et al.*, 2018; Feng *et al.*, 2022; Luo *et al.*, 2025). These cross-disciplinary contributions have established recombinant protein expression as a foundation of modern biotechnology innovation, with clear relevance in areas such as subunit vaccine development, including efforts by the Biomedical Laboratory of the Faculty of Veterinary Medicine at Universitas Udayana targeting strategic diseases such as African Swine Fever.

Conventional recombinant protein expression methods that rely on isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer in plasmid systems containing the lac operon face several significant limitations. These include toxicity that can inhibit cell growth, metabolic instability that may lead to the formation of inclusion bodies, and relatively high costs (Dvorak *et al.*, 2015; Gaglione *et al.*, 2019; Gomes *et al.*, 2020). For this reason, identifying alternative inducers that are safer, more economical, and capable of supporting optimal protein expression is essential. Lactose and skim milk have emerged as promising options because they act through the natural lac operon mechanism in *E. coli*, making them more compatible with cellular metabolism while offering lower costs (Khani and Bagheri, 2020). The importance of developing such alternatives lies not only in improving efficiency and reducing production costs but also in their potential for large-scale application.

This study aims to evaluate the effectiveness of lactose and skim milk as alternatives to IPTG for the expression of the GST-A276R fusion protein, a candidate subunit vaccine for African Swine Fever. The study was specifically designed to analyze and compare the effects of different inducer concentrations (1%, 2.5%, and 5%) and induction times (5, 24, and 48 hours)

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across the three types of inducers on the expression intensity of GST-A276R, in order to determine the most optimal induction conditions.

RESEARCH METHODS

Ethical Clearance for Animal Use

This study did not require animal ethics approval because all procedures were conducted entirely in vitro, without the use, handling, or intervention involving live animals.

Research Object

The research object was the recombinant GST-A276R protein expressed in the bacterial host system *E. coli* BL21. This protein was produced from the genetic construct of the plasmid pGEX-2TK-A276R. The A276R gene from the African Swine Fever virus was inserted into the pGEX-2TK expression vector under the control of the lac promoter.

Research Design

This study was a laboratory-based experimental study designed using a randomized block design. Induction time (5, 24, and 48 hours) was treated as the blocking factor to control variation associated with different bacterial growth phases. The experiment consisted of four treatment groups. The first group was a negative control, consisting of *E. coli* BL21 cultures without plasmid and without inducer in terrific broth glycerol (TBG) medium. The second group was a positive control, consisting of *E. coli* BL21 cultures carrying the pGEX-2TK-A276R plasmid and induced with 0.1 mM IPTG in TBG medium. The third group consisted of *E. coli* BL21 cultures carrying the pGEX-2TK-A276R plasmid induced with lactose at concentrations of 1%, 2.5%, and 5% in terrific broth (TB) medium without glycerol. The fourth group consisted of *E. coli* BL21 cultures carrying the pGEX-2TK-A276R plasmid induced with skim milk at concentrations of 1%, 2.5%, and 5% in TB medium.

A total of 72 samples were included in this study, comprising 9 negative control samples, 9 IPTG positive control samples, 27 lactose-treated samples, and 27 skim milk-treated samples. The 9 samples in both the negative and IPTG control groups were obtained from three time points (5, 24, and 48 hours), each with three replicates. The lactose and skim milk groups each consisted of three concentrations (1%, 2.5%, and 5%), evaluated at three time points with three replicates for each treatment combination.

Research Variables

The independent variables included the type of inducer (IPTG, lactose, and skim milk), with concentrations of 1%, 2.5%, and 5% for lactose and skim milk, and 0.1 mM for IPTG. Induction time was also an independent variable, with three observation points at 5, 24, and 48 hours. The dependent variable was the expression intensity of the recombinant GST-A276R protein, measured using the dot blot method. Controlled variables included incubation temperature and the type of bacterial growth medium to ensure consistent experimental conditions.

Preparation of Terrific Broth (TB) and Terrific Broth Glycerol (TBG)

TB and TBG media were prepared using two sterile 500 mL Schott Duran bottles, each filled with 500 mL of distilled water. For each bottle, 23.5 g of TB powder was added according to the manufacturer's instructions for 500 mL culture (Invitrogen). The media were homogenized by gently shaking the bottles until fully dissolved. For TBG preparation, 2 mL of glucose was added after homogenization. The solutions were then heated in a microwave for 30 minutes until boiling, followed by incubation at room temperature for 30 minutes. Sterilization was performed using an autoclave at 121 °C for 15 minutes.

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Preparation of Lactose and Skim Milk Inducers

Lactose and skim milk inducers were prepared using two sterile 500 mL Schott Duran bottles, each containing 500 mL of distilled water. Each solution was supplemented with 125 g of lactose or skim milk powder and homogenized by gentle shaking until fully dissolved. The solutions were heated in a microwave for 30 minutes until boiling, incubated at room temperature for 30 minutes, and sterilized using an autoclave at 121 °C for 15 minutes. Working concentrations of 1%, 2.5%, and 5% were obtained by diluting the stock solutions.

GST-A276R Culture

GST-A276R cultures were prepared using 6 mL of TBG medium supplemented with 60 µL of ampicillin (10 mg/mL) and 2 µL of *E. coli* BL21 carrying the pGEX-2TK-A276R plasmid. Samples were incubated for 48 hours at 37 °C with shaking at 120 rpm using an incubator shaker. Culture density was subsequently measured using a digital refractometer.

Induction Using Lactose and Skim Milk

Induction was performed in blue-cap tubes with a total sample volume of 3 mL derived from cultures previously propagated in TBG medium. The cultures were centrifuged to remove the supernatant, leaving only the bacterial cell pellet. The pellet was then resuspended in medium containing the appropriate inducer according to each treatment.

The volume of inducer added to each sample was calculated using a dilution formula to achieve the desired final concentration. The composition of each treatment was as follows: the negative control consisted of 3 mL of TBG medium, while the positive control consisted of 2977 µL TBG supplemented with 30 µL ampicillin and 3 µL IPTG. For the lactose treatment, samples consisted of 2880 µL TB supplemented with 30 µL ampicillin and 120 µL lactose for the 1% concentration, 2700 µL TB supplemented with 30 µL ampicillin and 300 µL lactose for the 2.5% concentration, and 2400 µL TB supplemented with 30 µL ampicillin and 600 µL lactose for the 5% concentration. For the skim milk treatment, samples consisted of 2880 µL TB supplemented with 30 µL ampicillin and 120 µL skim milk for the 1% concentration, 2700 µL TB supplemented with 30 µL ampicillin and 300 µL skim milk for the 2.5% concentration, and 2400 µL TB supplemented with 30 µL ampicillin and 600 µL skim milk for the 5% concentration. From each sample, 500 µL was collected at incubation times of 5, 24, and 48 hours and stored in a freezer for further analysis.

Confirmation of Protein Expression by Dot Blot

Frozen samples were thawed and 15 µL of each sample was mixed with 5 µL of buffer, then boiled at 100 °C for 8 minutes following the protocol of the Biomedical Laboratory, Faculty of Veterinary Medicine, Universitas Udayana. A volume of 10 µL from each sample was then spotted onto a nitrocellulose membrane. The membrane was incubated in a blocking solution prepared from 2 g of milk powder dissolved in 40 mL phosphate buffered saline with Tween (PBST) for 1 hour to prevent nonspecific binding. The membrane was then washed three times for 5 minutes each with PBST.

Next, the membrane was incubated with an anti-GST solution prepared at a 1:100 dilution in PBST (40 µL anti-GST in 40 mL PBST) for 1 hour, followed by three washes of 5 minutes each. The membrane was subsequently incubated with an anti-mouse secondary antibody at the same dilution for 1 hour and washed again three times for 5 minutes each.

In the final step, the membrane was incubated in a substrate solution with a total volume of 48 mL, consisting of 500 µL reagent A, 500 µL reagent B, and 200 µL AP color, to develop the protein expression signal. Dot blot intensity was analyzed using ImageJ software.

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Quantification of Dot Blot Signal Intensity Using ImageJ

Dot blot signal intensity was quantified using ImageJ software (NIH, USA). Each dot blot result was documented under uniform lighting conditions using a smartphone camera to ensure consistent contrast across samples. The images were imported into ImageJ and converted to 8-bit grayscale format to facilitate optical density measurement. Each protein signal was defined using a circular region of interest (ROI), and intensity values were measured using the integrated density parameter. Higher density values indicated stronger recombinant protein expression.

Data Analysis

Protein expression intensity data from all samples were compiled into tables and analyzed using IBM SPSS Statistics for Windows version 25. Statistical analysis began with the Shapiro-Wilk normality test to assess data distribution within each treatment group. This was followed by a two-way analysis of variance (ANOVA) using a factorial model with inducer concentration and induction time as factors, to evaluate their main effects and interaction on GST-A276R expression intensity. When significant differences were detected ($p < 0.05$), Tukey's Honest Significant Difference post hoc test was performed to identify specific differences between treatment means. The level of significance for all statistical tests was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Results

The *E. coli* BL21 culture carrying the pGEX-2TK-A276R plasmid was successfully grown in the culture medium. The culture exhibited normal growth with relatively homogeneous turbidity across samples, as shown in Figure 1. Expression of the recombinant GST-A276R protein was analyzed using the dot blot method by observing signal intensity across treatments. The dot blot results for all treatments are presented in Figure 2, where signals appear as spots with varying intensities among treatments, while no signal was detected in the control.

The left panel shows the results of induction using lactose, skim milk, and the negative control at three different induction times of 5, 24, and 48 hours. At 5 hours (rows A, B, and C), lactose treatments are shown in row A and part of row B, with 1% lactose at positions A4–A6, 2.5% lactose at A7–A9, and 5% lactose at B1–B3. Skim milk treatments are shown with 1% at B4–B6, 2.5% at B7–B9, and 5% at B10–B12. The negative control without inducer is located at C1–C3. Visually, at 5 hours, skim milk at 2.5% showed the strongest signal intensity, followed by skim milk at 1%.

At 24 hours (rows D, E, and F), the distribution pattern of treatments was similar to that at 5 hours. The signal intensity for skim milk at 2.5% remained more prominent than other treatments, while the control, lactose, and other skim milk concentrations showed weaker signals.

At 48 hours (rows G, H, and I), lactose treatments are shown in row G, with 1% lactose at G1–G3, 2.5% at G4–G6, and 5% at G7–G9. Skim milk treatments are shown in row H, with 1% at H1–H3, 2.5% at H4–H6, and 5% at H7–H9. The negative control is shown in row I at positions I1–I3. The strongest signal was observed in the 2.5% skim milk treatment, with spots appearing darker and more distinct compared to other treatments. Skim milk at 1% also showed increased intensity, whereas lactose treatments and the control remained relatively weaker.

The right panel shows dot blot results using 0.1 mM IPTG at 5 hours (J1–J3), 24 hours (K1–K3), and 48 hours (L1–L3), each with three replicates. Visually, IPTG produced detectable signals at all induction times, but the intensity appeared lower than that of 2.5% skim milk,

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particularly at 48 hours. The dot blot assays in the left and right panels were performed separately at different times.

Quantitative analysis of dot blot intensity for recombinant GST-A276R expression using ImageJ, presented as mean and standard deviation across inducer treatments and induction times, is shown in Table 1. The table indicates variation in intensity values across inducer types, concentrations, and induction times. The control group showed low and relatively uniform intensity values. Skim milk treatments produced higher intensity values than IPTG and lactose, with the highest mean consistently observed at 2.5% skim milk across all induction times. Lactose and IPTG treatments showed lower intensity values with relatively small variation across concentrations. Standard deviation values varied among treatments, reflecting data dispersion within replicates. Protein expression patterns across treatments are further visualized in the graph in Figure 3, which is organized by inducer concentration and induction time, with 5 hours shown in red, 24 hours in green, and 48 hours in blue.

The Shapiro–Wilk normality test in Table 2 showed that all treatment groups followed a normal distribution ($p > 0.05$), except for the negative control group, which showed a significance value below 0.05 ($p < 0.05$). The results of the two-way ANOVA for dot blot intensity of GST-A276R expression based on inducer concentration and induction time are presented in Table 3. The analysis showed that inducer concentration had a highly significant effect on protein expression ($F = 20.933$; $p < 0.001$). Induction time also had a highly significant effect ($F = 10.142$; $p < 0.001$). In addition, there was a highly significant interaction between inducer concentration and induction time ($F = 7.089$; $p < 0.001$).

Post hoc analysis using Tukey HSD, presented in Table 4, showed that the highest mean intensity was observed in the 2.5% skim milk treatment, particularly at 48 hours, which differed significantly from most other treatments. The 1% skim milk treatment also showed relatively high mean values and, at some induction times, fell within the same or adjacent significance groupings. In contrast, the control, IPTG, all lactose treatments, and several other skim milk treatments were grouped within the same notation and did not differ significantly from one another ($p > 0.05$).

Discussion

Recombinant protein production represents a fundamental pillar of modern biotechnology, supporting both basic research and practical applications such as vaccine development, diagnostics, and industrial bioproducts (Ferrer-Miralles *et al.*, 2009). Although *E. coli* expression systems are widely used due to their simplicity and efficiency, achieving stable and high protein yields still depends on proper optimization of the induction process (Rosano and Ceccarelli, 2014). Therefore, investigating induction parameters such as inducer type, concentration, and induction time is highly relevant for optimizing recombinant protein production and understanding lac operon regulation (Huang *et al.*, 2012). This study focuses on the recombinant GST-A276R protein as a candidate subunit vaccine against African Swine Fever, selected based on the strategic role of the A276R protein in modulating host immune responses (Correia, 2013). From an academic perspective, this study contributes quantitative validation of the specific effects of inducer type, induction time, and their interaction. From an applied perspective, it offers a practical approach to improving recombinant protein production efficiency by identifying safer and more cost-effective alternatives to IPTG, which is known to be toxic and expensive (Dvorak *et al.*, 2015).

Prior to protein expression analysis, the growth of *E. coli* BL21 cultures carrying the GST-A276R plasmid was evaluated. All treatments, including lactose, skim milk, IPTG, and the no-inducer control, exhibited colony growth without noticeable differences, indicating that cells

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remained viable during induction. Recombinant protein production in *E. coli* depends on the balance between induction strength and the cell's ability to sustain growth, as excessive induction can impose metabolic burden and reduce cellular stability (Rajacharya *et al.*, 2024; Rosano and Ceccarelli, 2014). The relatively uniform colony growth observed in this study suggests that variations in GST-A276R expression were primarily influenced by inducer effectiveness rather than differences in cell growth.

Protein expression was then assessed using the dot blot method. Visualization revealed detectable protein signals in all induced treatments, with varying intensities across concentrations and induction times, while the negative control showed no signal. Differences in spot thickness and darkness reflect variations in expression levels, as signal intensity correlates with the relative amount of detected protein (Častorálová *et al.*, 2012; Lu *et al.*, 2026). Dot blot is an immunodetection technique based on antigen-antibody interactions applied directly to membranes without electrophoretic separation, making it suitable for rapid analysis and relative comparison of protein expression across samples (Mishra, 2022). Variation in signal intensity across different combinations of inducer concentration and induction time indicates that induction conditions strongly influence recombinant protein production, consistent with previous reports on *E. coli* expression systems (Rosano and Ceccarelli, 2014). Signal intensity was subsequently quantified using ImageJ, a method with sufficient sensitivity to detect expression differences among treatments (Puzari *et al.*, 2025; Zhuang *et al.*, 2025). Quantification involved image inversion, background correction, and integrated density measurement using consistent selection areas to ensure objective comparison across treatments.

The dynamics of induction time showed a non-linear pattern, with relatively high expression at 5 hours, a decrease at 24 hours, and an increase again at 48 hours. At the early 5-hour time point, cells were likely still in the exponential phase, with optimal translational capacity supporting efficient recombinant protein production (Baneyx, 1999; Shiloach and Fass, 2005). The decrease at 24 hours may be attributed to the accumulation of metabolic burden and potential toxicity of the A276R gene, which can induce cellular stress, slow growth, and increase protein degradation or inclusion body formation (Lozano Terol *et al.*, 2021; Rosano and Ceccarelli, 2014). The increase observed at 48 hours may reflect physiological adaptation of the cells to expression stress. This pattern demonstrates that recombinant protein expression does not necessarily increase linearly over time, but is influenced by the balance between cell growth, metabolic burden, and target protein stability (Baneyx, 1999; Rosano and Ceccarelli, 2014).

The Shapiro–Wilk normality test indicated that most treatment groups were normally distributed, while the control group showed a significance value below 0.05. Nevertheless, two-way ANOVA remained appropriate for evaluating the effects of inducer concentration and induction time, as this method is robust to mild violations of normality (Blanca, 2017). Statistical analysis confirmed that inducer concentration, induction time, and their interaction significantly affected GST-A276R expression intensity ($p < 0.001$). These findings reinforce the understanding that recombinant protein expression is a multifactorial process. Optimization depends on the dynamic interaction between external induction parameters and the internal physiological state of the host cells (Gomes *et al.*, 2020; Sørensen and Mortensen, 2005). Tukey HSD post hoc analysis revealed significant differences among treatment combinations, explaining why protein expression did not increase linearly over time. This phenomenon reflects a complex cellular balance among translational capacity, inducer availability, and metabolic conditions in *E. coli* BL21 during induction (Baneyx, 1999; Rosano and Ceccarelli, 2014).

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Based on the data analysis, induction with 2.5% skim milk produced the highest GST-A276R expression intensity and differed significantly from other treatments. This was followed by 1% skim milk, which also demonstrated strong expression performance. These findings extend previous work by Khani and Bagheri (2020), which reported the effectiveness of skim milk as an alternative to IPTG. Theoretically, a moderate inducer concentration provides an optimal balance between activation of the lac promoter and cellular translational capacity, thereby reducing excessive metabolic burden in *E. coli* BL21 (Gomes *et al.*, 2020; Rosano and Ceccarelli, 2014).

The superiority of skim milk as an inducer is likely associated with two main factors. First, its lactose content acts as a natural inducer of the lac operon system. Second, additional nutritional components such as proteins and minerals in skim milk may support cellular metabolic stability during induction (Alsaleem *et al.*, 2023; Khani and Bagheri, 2020). This combination enables more controlled and sustained protein expression compared to IPTG. Synthetic inducers such as IPTG may impose metabolic stress that can interfere with the expression process (Dvorak *et al.*, 2015).

Variation in lactose concentration (1%, 2.5%, and 5%) and standard induction using 0.1 mM IPTG resulted in lower expression intensities and did not show significant differences among treatments. Lactose, as a natural inducer, must first be converted into allolactose to bind the LacI repressor and release it from the operator, whereas IPTG, as a non-metabolizable analog, can directly activate gene expression because it is not degraded within the cell (Juers *et al.*, 2012; Lewis, 2005). This mechanism leads to weaker induction by lactose and makes it highly dependent on the physiological state of the cell, as it operates through the natural regulation of the lac system with lower affinity for the repressor compared to IPTG (Marbach and Bettenbrock, 2012).

Although IPTG is widely recognized as a strong inducer of the lac operon system, its application in this study did not result in higher protein expression. This pattern is consistent with several studies reporting that IPTG can increase metabolic burden and trigger cellular stress due to high levels of recombinant protein expression, particularly when the target protein is toxic or disrupts cellular homeostasis (Gomes *et al.*, 2020; Rizkia *et al.*, 2015). In addition, if the A276R gene exerts mild toxicity, cells may limit their translational capacity as an adaptive response to expression stress, resulting in relatively similar and non-significant expression levels across all lactose treatments (James *et al.*, 2021). The low protein expression observed with 0.1 mM IPTG in this study is also consistent with previous reports. Lozano Terol *et al.* (2021) demonstrated that in lac-based systems, maximal expression is often achieved at 0.1 mM IPTG, and increasing the concentration up to 2 mM does not significantly enhance final expression levels but mainly affects early expression kinetics. Similarly, Larentis *et al.* (2014) reported that varying IPTG concentrations within the range of 0.1 to 1 mM did not improve expression when normalized to cell growth, and higher concentrations negatively affected growth and increased the fraction of insoluble protein. These findings suggest that 0.1 mM IPTG generally represents an effective induction level in lac systems, and the lower expression observed in this study likely reflects physiological limitations of the expression system or adaptive cellular responses to metabolic burden rather than a failure of induction.

The results of this study specifically indicate that a skim milk concentration of 2.5% provides the most balanced induction condition. At this concentration, activation of the expression system reaches an optimal level without imposing excessive metabolic stress on the cells (Amoozadeh, 2018; Pugliese *et al.*, 2017). In contrast, increasing the concentration to 5% begins to show limiting effects. Factors such as increased medium viscosity or nutrient imbalance may contribute to reduced expression efficiency, although signal intensity at this

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concentration remains relatively high (Khani and Bagheri, 2020). By comparison, lactose at concentrations of 1% to 5% resulted in low expression levels that were not significantly different from the 0.1 mM IPTG control. This finding differs from Seung-Hyun *et al.* (2020), who reported optimal GFP expression at lactose concentrations of 2.6 to 3.4%. This discrepancy suggests that responses to lactose may be highly specific, influenced by factors such as cell permeability, inducer metabolism rate, and the affinity of the regulatory system for specific target proteins (Lewis, 2005; Reznikoff, 1992). The relatively low expression observed with 0.1 mM IPTG further supports studies highlighting the limitations of this synthetic inducer. These findings are consistent with Dvorak *et al.* (2015) and Gomes *et al.* (2020), who reported that IPTG can induce metabolic stress, suppress cell growth, and ultimately limit protein productivity, especially in prolonged cultures. Therefore, although IPTG is considered a standard inducer, its effectiveness is not universal and can be surpassed by alternative inducers under certain conditions.

Based on these findings, this study confirms the importance of developing induction protocols that are consistent, cost-effective, and easy to implement as alternatives to IPTG-dependent systems. Skim milk fulfills these criteria and demonstrated superior induction performance compared to both IPTG and lactose. This indicates that skim milk-based protocols have strong potential for application across different scales, from laboratory settings to industrial recombinant protein production. Standardizing induction protocols using simple and accessible materials may support the sustainability of research and the broader application of recombinant protein biotechnology.

Despite these findings, several limitations should be acknowledged. Protein expression analysis was performed using dot blot, which is a qualitative method and does not provide precise quantification of absolute protein levels or the proportion of soluble and insoluble fractions (Mishra, 2022). The study also did not evaluate functional aspects of the protein, such as folding, stability, and biological activity. In addition, culture parameters such as growth phase at induction, induction temperature, and cell density were not specifically optimized, which may have influenced the overall expression outcomes.

Overall, this study highlights that the interaction between inducer concentration and induction time is a key determinant of successful GST-A276R recombinant protein expression in *E. coli* BL21. These findings provide a solid scientific basis for developing more efficient and cost-effective induction protocols. However, interpretation of the results should consider the methodological limitations. The findings should be regarded as a valuable starting point for further studies aimed at validating and refining induction protocols for broader and more reliable production applications.

CONCLUSION AND SUGGESTIONS

Conclusion

The type of inducer had a highly significant effect on the expression level of the GST-A276R protein ($p < 0.05$). The combination of 2.5% skim milk and a 48-hour induction time represented the most optimal condition for GST-A276R expression. Inducer concentration, both lactose and skim milk, as well as induction time, also had a significant effect on GST-A276R protein expression ($p < 0.05$).

Suggestions

For further development, this study recommends evaluating the functional quality and biological activity of the GST-A276R protein expressed under optimal conditions using 2.5% skim milk. Optimization of culture parameters, such as optical density at the time of induction

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and incubation temperature, may further improve protein yield. Validation of the skim milk–based induction protocol at a larger culture scale is necessary to assess its feasibility for industrial production. In addition, immunogenicity testing of the optimally expressed protein in animal models is required to confirm its potential as an effective ASF subunit vaccine candidate.

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Figures

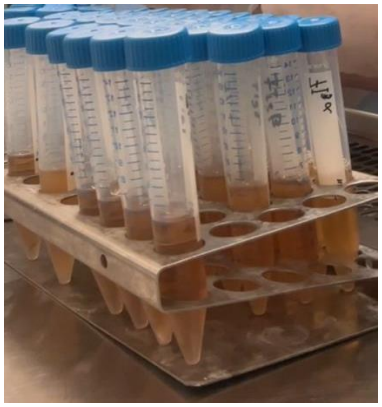

Figure 1. *E. coli* BL21 bacterial culture harboring the pGEX-2TK-A276R plasmid

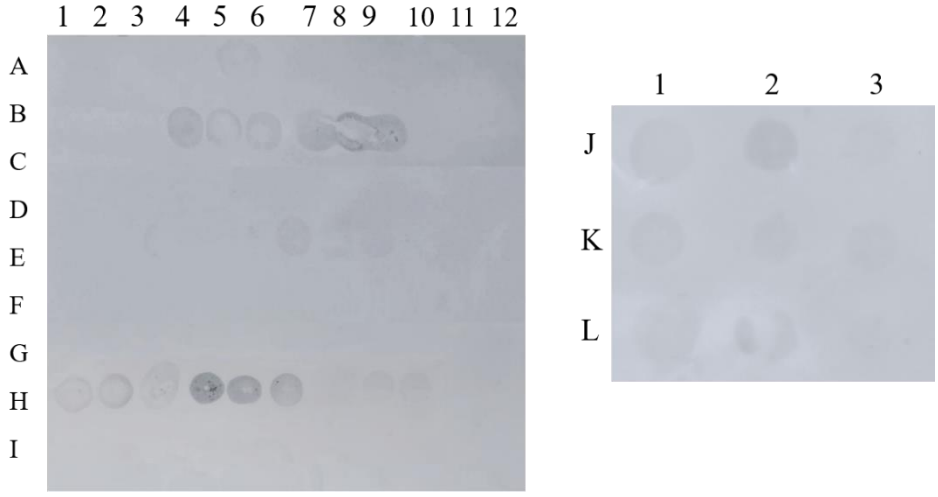

Figure 2. Dot blot results of GST-A276R protein induction using lactose (rows A, D, G), skim milk (rows B, E, H), and control (rows C, F, I) at three induction times: 5 hours (A, B, C), 24 hours (D, E, F), and 48 hours (G, H, I). The right panel shows dot blot results using 0.1 mM IPTG at 5 hours (J), 24 hours (K), and 48 hours (L1–L3), each with three replicates. Dot blot assays in the left and right panels were performed separately at different times.

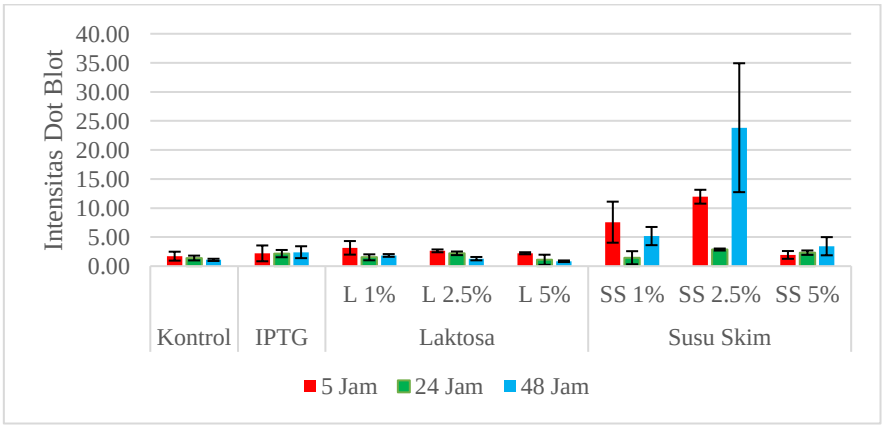

Figure 3. Graph of mean dot blot intensity quantified using ImageJ for recombinant GST-A276R protein expression based on variations in inducer concentration and induction time.

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Tables

Table 1. Mean and standard deviation (SD) values of recombinant GST-A276R protein expression intensity based on dot blot quantification using ImageJ across different inducers, concentrations, and induction times.

Induction time	Replication	INDUCTOR							
		Control	IPTG	Lactose			Skim Milk		
				1%	2.5%	5%	1%	2.5%	5%
5	1	0.92	1.20	2.94	2.87	2.05	11.6	10.62	1.31
	2	2.88	3.75	4.41	2.38	2.20	5.02	12.27	1.83
	3	1.36	1.65	2.10	2.64	2.40	6.08	12.94	2.64
	Mean SD	1.72	2.20	3.15	2.63	2.22	7.57	11.95	1.93
		0.77	1.36	1.17	0.25	0.18	3.53	1.19	0.67
24	1	1.84	2.64	1.08	1.98	1.95	0.77	3.01	2.34
	2	1.02	2.38	1.39	2.09	0.21	0.84	2.91	2.69
	3	0.90	1.45	2.08	2.56	1.14	275	2.67	1.93
	Mean SD	1.40	2.16	1.52	2.21	1.10	1.45	2.86	2.32
		0.41	0.63	0.51	0.31	0.87	1.12	0.17	0.38
48	1	0.92	1.75	2.11	0.99	0.94	3.82	35.65	1.72
	2	1.02	3.58	1.68	1.30	0.68	6.89	22.18	4.78
	3	1.29	1.87	1.73	1.57	0.90	4.82	13.65	3.80
	Mean SD	1.08	2.40	1.84	1.29	0.84	5.18	23.83	3.43
		0.19	1.02	0.24	0.29	1.57	1.57	11.09	1.56

Note: Intensity values represent measurements after background subtraction. Data are presented as mean ± standard deviation (n = 3).

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Table 2. Results of the Shapiro–Wilk normality test for GST-A276R protein expression values across treatment groups.

Treatment	Statistic	Signification (p > 0.05)	Remarks
Negative Control	0.764	0.008	Abnormal
IPTG 0.1 mM	0.896	0.232	Normal
Lactose 1%	0.849	0.073	Normal
Lactose 1%	0.849	0.073	Normal
Lactose 2.5%	0.952	0.710	Normal
Lactose 5%	0.919	0.383	Normal
Skim Milk 1%	0.929	0.474	Normal
Skim Milk 2.5%	0.870	0.123	Normal
Skim Milk 5%	0.897	0.235	Normal

Table 3. Two-way ANOVA of mean dot blot intensity measured using ImageJ to evaluate the effects of inducer concentration and induction time on recombinant GST-A276R protein expression.

Tested factors	F value	p-*value* (p < 0.001)
Inductor	20.933	< 0.000
Time	10.142	< 0.000
Time × Inductor	7.089	< 0.000

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Table 4. Tukey HSD post hoc test results for mean dot blot intensity of recombinant GST-A276R protein expression based on variations in inducer concentration and induction time.

Treatment	Concentration	Time	Mean	SD	Notation
Control (-)	-	5 hours	1.72	0.77	a
Control (-)	-	24 hours	1.40	0.41	a
Control (-)	-	48 hours	1.08	0.19	a
IPTG	0.1 mM	5 hours	2.20	1.36	a
IPTG	0.1 mM	24 hours	2.16	0.63	a
IPTG	0.1 mM	48 hours	2.40	1.02	a
Lactose	1%	5 hours	3.15	1.17	a
Lactose	1%	24 hours	1.52	0.51	a
Lactose	1%	48 hours	1.84	0.24	a
Lactose	2.5%	5 hours	2.63	0.25	a
Lactose	2.5%	24 hours	2.21	0.31	a
Lactose	2.5%	48 hours	1.29	0.29	a
Lactose	5%	5 hours	2.22	0.18	a
Lactose	5%	24 hours	1.10	0.87	a
Lactose	5%	48 hours	0.84	1.57	a
Skim Milk	1%	5 hours	7.57	3.53	ab
Skim Milk	1%	24 hours	1.45	1.12	a
Skim Milk	1%	48 hours	5.18	1.57	ab
Skim Milk	2.5%	5 hours	11.95	1.19	b
Skim Milk	2.5%	24 hours	2.86	0.17	a
Skim Milk	2.5%	48 hours	23.83	11.09	c
Skim Milk	5%	5 hours	1.93	0.67	a
Skim Milk	5%	24 hours	2.32	0.38	a
Skim Milk	5%	48 hours	3.43	1.56	a

Note: Interpretation is based on letter notation. Identical letters indicate no significant difference, while different letters indicate significant differences among treatments ($\alpha = 0.05$).

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