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In Silico Design and Expression of Dengue Virus Type 2 Envelope Domain III in *Escherichia coli* BL21 (DE3) for Specific Diagnostics

[1,2]Gaspary Mwanyika
[1]Department of Applied Sciences, Mbeya University of Science and Technology, P.O Box 131 Mbeya, Tanzania
[2]Centre for Epidemic Response and Innovation (CERI), School for Data Science and Computational Thinking, Stellenbosch University, Stellenbosch 7600, South Africa
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ABSTRACT

Dengue virus type 2 (DENV-2) is a mosquito-borne virus of global public health concern. Routine diagnostic tests often target whole envelope glycoproteins that suffer from cross-reactivity. The envelope domain III (EDIII) protein contains subtype virus-specific epitopes, offering improved specificity over complete envelope. This study evaluated a modified DENV-2 EDIII expression in E. coli BL21 (DE3) using a codon-optimized construct. AlphaFold2 algorithms were utilized to predict its 3D structure and identify potential antigenic epitopes. The construct was stable (instability index <40), soluble and antigenic (>0.5score, respectively) containi ng three key linear epitopes; CTGKFKVVKEIAETQH (302–317aa), SPCKIPFEIMDLEKR (331–345aa), and QLKLNWFKKGSS (386–396aa). Optimal expression occurred at 30°C and 4 hours post-induction, producing a distinct 14 kDa band on SDS-PAGE consistent with the expected molecular weight of target EDIII protein. Overall, these findings demonstrate that in silico protein design with low-temperature bacterial expression can produce recombinant proteins suitable for specific diagnostics.

*Corresponding author's e-mail address: mwanyikag254@gmail.com (Mwanyika, G.)

1.0 Introduction

Dengue is a major mosquito-borne viral disease of global public health concern. The disease is endemic in more than 100 countries, causing approximately 400 million infections and 22,000 deaths annually (Roy & Bhattacharjee, 2021). In the past decade, the global incidence of dengue has increased significantly, with nearly 40% of the world's population at risk of dengue virus (DENV) infection (Souza-Neto *et al.,* 2019).

The recent surge in DENV infections is exacerbated by increased global connectivity through human mobility, urbanisation, and environmental suitability due to climate change (Harish *et al.,* 2024). The disease is caused by a single-stranded RNA virus of the family *Flaviviridae* in the genus Flavivirus (Liu *et al.,* 2023). The virus is transmitted in humans through the bites of infected female Aedes mosquito species (Muktar *et al.,* 2016). Among the four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), DENV-2 causes one-third of the global dengue disease burden and is frequently associated with severe diseases (Soo *et al.,* 2016). The absence of effective vaccines and specific antiviral therapy against DENV infection continues to pose a public health threat. Reliance on a clinical diagnosis is complex because symptoms often overlap with other febrile illnesses, such as malaria, chikungunya, typhoid fever, and influenza. Consequently, early detection and vector control remain primary strategies for dengue prevention and management (Guzman & Harris, 2015).

The dengue RNA genome encodes three structural proteins: capsid-C, precursor membrane (prM) and envelope (E) and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Kalimuddin *et al.,* 2025). The E protein is a key structural protein containing 450 amino acid residues (aa) that mediates viral entry and is a target for neutralising antibodies. These features make it a good target for diagnostic and vaccine development (Fig. 1). The E protein has three structural immunoglobulin-like domains, EDI, EDII, and EDIII. EDIII spans from 300 to 400aa of the envelop protein c-terminus and contains numerous subtype and virus-specific epitopes (Fahimi *et al.,* 2018; X. Zhang *et al.,* 2017). Additionally, it lacks immunodominant cross-reactive epitopes (Kim *et al.,* 2016), making it a suitable diagnostic biomarker for serotype-specific dengue detection assays (Hou et al., 2022).

The current dengue diagnostic methods, such as rapid serological assays, face several limitations, including cross-reactivity with closely related flaviviruses and variable sensitivity. Molecular diagnostic tests such as Reverse transcriptase Polymerase Chain Reaction (RT-PCR) require high-cost reagents, advanced laboratory facilities and skilled personnel (Selvaraj *et al.,* 2025). Therefore, the development of recombinant immunodiagnostic antigens that are subtype-specific and easy to produce is crucial for the advancement of dengue rapid diagnostics. *In silico* design to enhance the physicochemical properties of the target envelope domain III (EDIII) protein offers an efficient strategy for laboratory validation, reducing both time and cost.

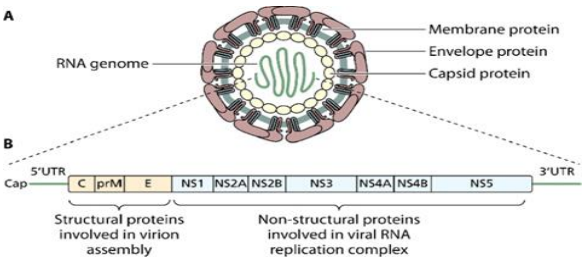
Few studies have integrated computational design of DENV-2 EDIII with optimised recombinant expression in simple bacterial systems such as *Escherichia coli BL21* (DE3). In recent years, recombinant EDIII protein has been purified from *Escherichia coli,* with many attempts resulting in the expression of insoluble or low-yield recombinant proteins (San-Miguel *et al.,* 2013a; Tennakoon *et al.,* 2025).

This study aimed to improve the physicochemical properties of the DENV-2 EDIII domain through *in silico* design and evaluate its expression in the *Escherichia coli* BL21 (DE3) host cells. The goal is to develop recombinant EDIII proteins suitable for use as immunodiagnostic antigens, ultimately supporting the development of serotype-specific dengue detection assays.

Figure 1

Schematic Representation Dengue Virus Genome

A. *Cross-Sectional View of DENV Virion*
B. *DENV RNA Genome Containing Three Structural Proteins and Seven Non-Structural Proteins With 5′ and 3′ Untranslated Regions (UTRS) (Kalimuddin et al., 2025)*



2.0 Materials and Methods

2.1 Experimental Materials

Escherichia coli BL21 (DE3) was used as the bacterial expression strain, pET22b (GeneScript, USA) expression vector with BamHI-5' and XhoI-3' restriction sites. Luria-Bertani (LB) broth (w/v) and LB agar (Sigma-Aldrich, USA) were used for culture, while ampicillin stock (Sigma-Aldrich, USA) was used for selection of bacterial transformants.

2.2 Sequence Retrieval

Amino acid sequences of EDIII protein representing dengue virus serotype 2 were retrieved from the National Centre for Biotechnology Information protein database (NCBI) (Table 1). The consensus sequence was created using BioEdit software v. 7.2 (Hall, 1999), and conservation was confirmed using the Sequence Manipulation Suite bioinformatics t ool at https://www.bioinformatics.org/sms2/color_align_cons.html (Stothard, 2000). The physicochemical properties of the target EDIII protein were predicted using the ProtParam prog ram implemented in the ExPASy Bioinformatics R esource Portal available at https://web.expasy.org/protparam/(Gasteiger *et al.,* 2005).

Table 1
Selected Dengue Virus Serotype 2 (DENV-2) Strains Used in this Study

Strainname	GenBank accession#
16681-PDK53	P2999.1
China/D2-04	P30026.1
Jamaica/1409/1983	P07564.2
Malaysia M2	P14338.1
Peru/IQT2912/1996	Q9WDA6.1
Thailand/16681/84	P29990.1

2.3 Physicochemical and Immunological Properties

The physicochemical and immunological properties of the envelope domain III protein were analysed using established bioinformatics tools. The molecular weight (MW), theoretical isoelectric point (pI), stability index, and grand average hydropathicity index were computed using EXPASy ProtParam (EXPASyProtParam (https://web.expasy.org) (Gasteiger *et al.,* 2005). The solubility of recombinant EDIII protein in *E. coli* was predicted using Protein-Sol, accessed at http://protein-sol.manchester.ac.uk (Hebditch *et al.,* 2017). The overall antigenicity score was estimated with VaxiJen application software v2.0 available at http://www.ddg-pharmfac.net/vaxijen/. (Doytchinova & Flower, 2007). The linear B-cell epitopes were predicted using the Bepipred linear epitope prediction tool v2.0, and the three-dimensional structure was predicted using Alpha Fold2 algorithms (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=G4yBrceuFbf3) (Mirdita *et al.,* 2022). Structural visualisation and annotation were performed using the PyMOL™ Molecular Graphics System v2.3.0 implemented in an Ubuntu Linux environment. Custom PyMOL scripts were utilised to render the protein surface and highlight epitope positions.

2.4 Plasmid Construction

The envelope domain III target protein sequence of dengue virus serotype 2 (D2EDIII) was retrieved from the Protein Data Bank (PDB ID 2JSF) accessed at http://www.rcsb.org/structure/2JSF. The target DENV-2EDIII domain is shown in Fig. 2. To enhance domain specificity, eight (8) amino acids (289-296 aa) were excluded, as they constitute part of domain I (281-296 aa) (Yang *et al.,* 2012) rather than EDIII. Additionally, 13 amino acids (400-412 aa) corresponding to an extended envelope stem region were incorporated to improve the structural stability of the domain (Fig. 3).

Figure 2
Improved DENV-2 Envelope Domain III Target

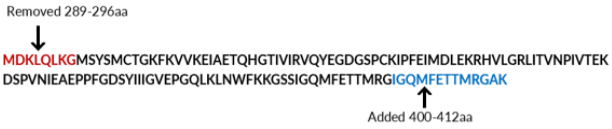
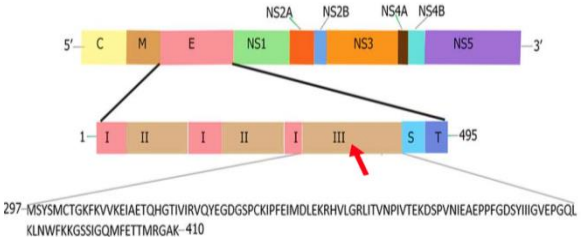


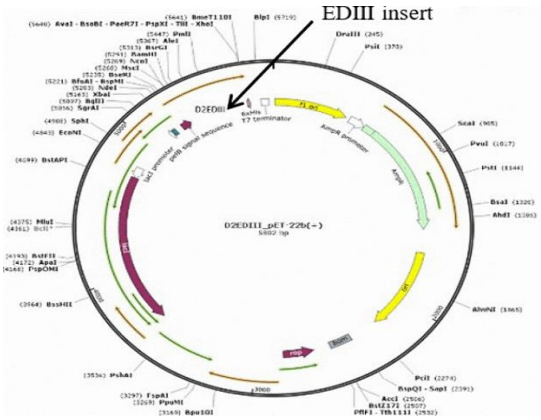
Figure 3
The Modified DENV-2 Envelope Domain III Target



To enhance expression efficiency in *Escherichia coli BL21* (DE3), the EDIII nucleotide sequence was optimised by correcting codons that are rarely used by *Escherichia coli* species using the GenScript Rare Codon Analysis Tool ac

cessed at https://www.genscript.com/tools/rare-codon-analysis. The optimised EDIII gene was commercially synthesised (GenScript Inc., USA) and cloned into the pET22b (+) expression vector using BamHI (5') and XhoI (3') restriction sites. The resulting expression cassette contained an N-terminal pectate lyase B (pelB) signal peptide to direct the recombinant protein into E. coli periplasm for secretion. Periplasmic space and a C-terminal hexahistidine (6xHis) tag to facilitate purification by immobilised metal affinity chromatography (IMAC) (Fig. 4).

Figure 4
Schematic Map of D2EDIII_pET22b (+) Expression Construct Developed in this Study



2.5 D2EDIII Expression and Analysis

Expression of D2EDIII was performed following the method previously described by Tennakoon *et al.* (2025) with minor modifications. The D2EDIII_pET22b plasmid was transformed into E. coli BL21 (DE3) cells. A single colony harbouring the target plasmid was selected and inoculated into 50 mL of Luria-Bertani (LB) supplemented with 100 µg/mL of ampicillin (Sigma, USA) and 0.5% glucose. Thereafter, it was grown overnight at 37°C with shaking at 200 rpm. The overnight starter culture was then transferred into 200 mL of fresh LB medium containing 100 µg/mL of ampicillin and incubated at 37 °C with shaking at 200 rpm until reaching mid-logarithmic growth (OD600 ~ 0.6). The culture was subsequently divided for induction at two different temperatures: 30 °C and 37 °C. Recombinant expression was induced by adding 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). Cultures were incubated at the respective induction temperatures with shaking at 225 rpm. Growth and protein expression were monitored by measuring OD600 every 2 hours up to 24 hours. Uninduced cultures were maintained as negative controls. Cells were finally harvested by centrifugation at 5,000 x g for 10 minutes, and the supernatant discarded. The resulting cell pellets were resuspended in 4x Laemmli buffer and subjected to Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 12% gel. Protein separation was performed at 75 V for 20 minutes for stacking, followed by 150 V for 70 minutes for resolving the proteins. Expression levels of recombinant EDIII were visualised on SDS-PAGE and analysed by comparing induced and uninduced culture samples with the intensity of corresponding bands.

3.0 Results

The consensus envelope protein domain III containing 114 amino acid residues was created from the amino acid sequences obtained from the NCBI database representing the DENV-2 strains (Fig. 5). *In silico* analysis showed that the target EDIII protein had a predicted molecular weight of 13.7 kDa, which is comparable to the reference DENV-2 (2JSF) EDIII protein (13.3 kDa, Mw). The protein exhibited a theoretical isoelectric point (pI) of 6.7 and was predicted to be stable, with a GRAVY index below 40 (Table 2). The solubility analysis showed that the target EDIII protein had a high probability of solubility (QuerySol = 0.558), which exceeded the population average solubility (PopAvrSol = 0.45) of E. coli proteins (Fig. 6A). Antigenicity analysis classified the EDIII protein as a probable antigen, with three predicted antigenic epitopes: CTGKFKVVKEIAETQH (300–317 aa) position, SPCKIPFEIMDLEKR (331–346 aa) and QLKLNWFKKGSS (386–397 aa) surpassing the threshold score of 0.5 (Fig. 6B).

The transformation of *Escherichia coli* BL21 (DE3) cells with the recombinant pET-22b(+) EDIII construct was confirmed by the successful growth of transformants on Luria-Bertani (LB) agar plates supplemented with ampicillin (Fig. 7A and B), indicating the retention of plasmids carrying ampicillin resistance markers.

Figure 5

Consensus DENV-2 Envelope Protein Domain III Target

Notes: The identical residues are marked with a black background, and those that are similar are shown with a gray color background.

Table 2

Predicted Physicochemical Properties of Target DENV-2 Envelope Domain III (D2EDIII) Protein

Physicochemical parameter	Values for target D2EDIII	Values for reference 2JSF EDIII
Molecular weight (MW)	13.7 kDa	13.3 kDa
Theoretical isoelectric point (pI)	6.7	6.7
Instability index*	38.8	39.3
Gravy**	-0.384	0.368

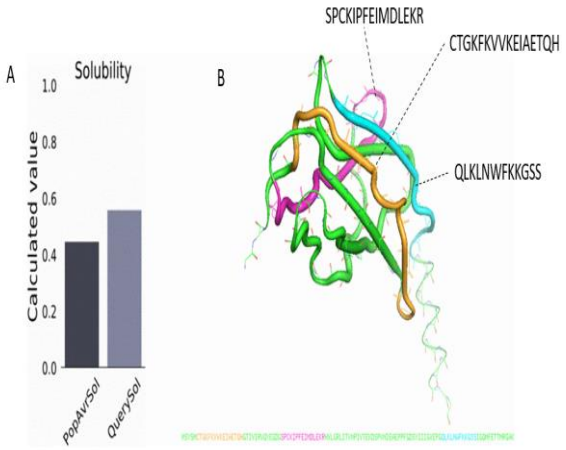
*Protein with an instability index < 40 is predicted being stable and > 40 as unstable

**Gravy (grand average of hydropathicity) index indicates the solubility of proteins; positive gravy refers to hydrophobicity and negative gravy refer to hydrophilicity.

Figure 6

A. Predicted Scaled Solubility of Target D2EDIII Protein (Querysol) Compared with Population Average Solubility of E. Coli Proteins (PopAverSol)

B. Predicted Epitopes on the Target DENV-2 EDIII Protein, CTGKFKVVKEIAETQH (in Orange), SPCKIPFEIMDLEKR (in Magenta) and QLKLNWFKKGSS (in Cyan)



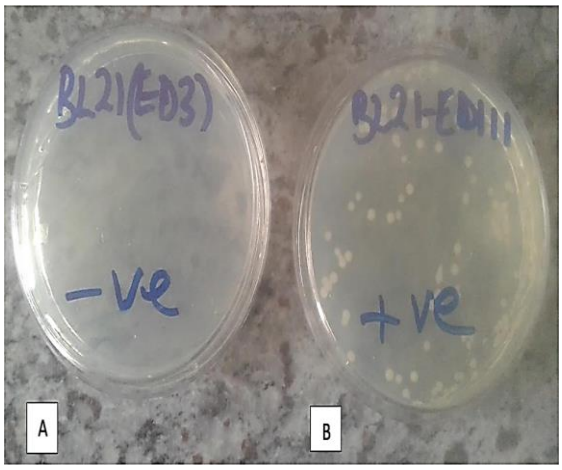
Notes: The epitopes were mapped and visualized using PyMOL™ Molecular Graphics Systemv2.3.0.

Figure 7

Selection of Transformants on LB-Ampicillin Plates

A. Untransformed E. Coli Cells Did Not Grow on LB-Ampicillin Agar (Negative Plates, -Ve)

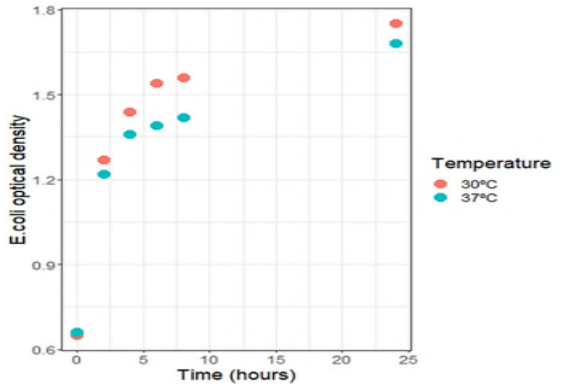
B. Transformed E. Coli Cells Contained Ampicillin Resistant Markers Grew on LB Ampicillin Agar (Positive Plates, +Ve)



Higher E. coli cell growth density was observed at 30 °C temperature conditions, suggesting that optimal expression of recombinant EDIII protein

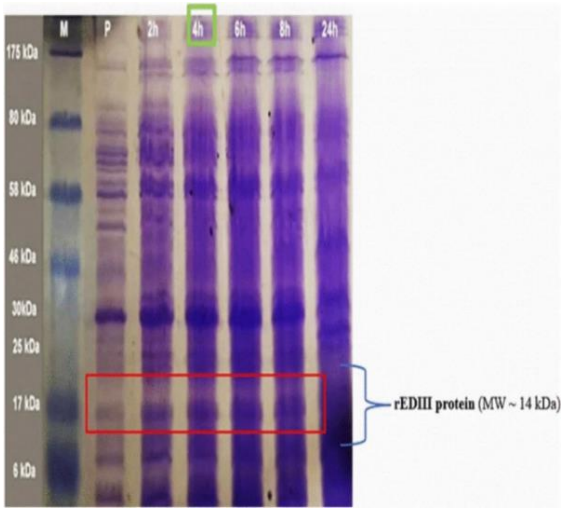
occurred when induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was performed at 30 °C compared to normal bacterial growth temperature of 37°C (Fig 8).

Figure 8
Effect of IPTG Induction at 30 °C and 37 °C Temperature Conditions on the Expression of Denv-2 EDIII Protein



SDS-PAGE analysis revealed prominent bands migrating below 17 kDa that match the expected molecular weight of the target EDIII protein (13.7 kDa). The presence and increased intensity of these bands in IPTG-induced cultures (2, 4, 6, and 8-hour cultures) and their absence in pre-induced culture samples (P) confirm a successful expression of the recombinant EDIII protein (Fig. 9).

Figure 9
SDS-PAGE Analysis of Recombinant DENV2 EDIII (rEDIII) Protein
M: Broad Range Protein Ladder,
P: Pre-Induced Culture, 2h, 4h, 6h, 8h and 24h Post-Induction Cultures



4.0 Discussion

This study aimed at designing a dengue virus serotype 2 EDIII construct and expressing it in the *Escherichia coli* BL21 (DE3) system. The system has been widely used for heterologous expression of recombinant proteins because it is a well-studied expression system, having a rapid growth rate and being cost-effective compared to eukaryotic expression systems such as yeast and mammalian cells (Hayat *et al.,* 2018; Jia & Jeon, 2016). Despite the advantages, there are several challenges in the production of heterologous recombinant proteins using bacterial expression systems, including improper folding of expressed proteins due to lack of correct disulphide bonding in the cytoplasm and the presence of rare codons (Gomes *et al.,* 2016; Kaur *et al.,* 2018). Production of recombinant EDIII protein of DENV-2 has been previously described (Libraty *et al.,* 2015; Nguyen *et al.,* 2019). However, expression of membrane proteins like the EDIII domain usually results in aggregated products known as inclusion bodies that are hard to purify (Pereira *et al.,* 2024; Tennakoon *et al.,* 2025).

To overcome this challenge, several strategies have been adopted, such as co-expression with solubility tags like maltose-binding protein (MBP) (Xiao *et al.,* 2018) and thioredoxin fusion protein (TrxA) (Rasooli & Hashemi, 2019), targeting periplasmic secretion (Karyolaimos & de Gier, 2021) and inducing expression at different temperature conditions (Tennakoon *et al.,* 2025). The use of solubility fusion tags may not be beneficial due to steric hindrance interference which can destruct the structure and biological activity of target proteins; in addition, the removal of fusion tags is relatively expensive and requires tedious cleavage optimisation conditions (Costa *et al.,* 2014).

In this study, *in silico* design and optimised culture strategies were integrated to enhance the physicochemical properties, correct rare codons used by *Escherichia coli* and optimise expression of the envelope protein domain III protein of DENV-2. The design led to the creation of a more stable (Table 2) and soluble construct (Fig. 6A). The presence of rare codons in tRNA can stop transcription and translation (Kane, 1995; Karlin *et al.,* 1998). The pET22b (+) vector used in the

present study carries the pectate lyase B (pelB) signal sequence originating from *Erwinia carotovora* for directing the expressed proteins into the bacteria's periplasm (Choi & Lee, 2004). Recombinant expression into the periplasm allows the correct disulphide bond formation of recombinant proteins for maintaining their native conformation, solubility and biological activity (Lokireddy *et al.,* 2025). Since proteins containing cystine bridges require an oxidation environment present in the periplasm for disulphide bond formation. In comparison, the expression of protein into the cytoplasm could cause improper folding and aggregation of expressed proteins because the cytoplasm is a reducing environment that does not support the formation of disulphide bonds (Landeta *et al.,* 2018; Saaranen & Ruddock, 2013).

Antigenic characterisation revealed three epitopes, namely CTGKFKVVKEIAETQH (300–317 aa), SPCKIPFEIMDLEKR (331–346 aa), and QLKLNWFKKGSS (386–397 aa) (Fig. 6B). These epitopes concur with known antigenic hotspots within the DENV-2 EDIII domain (Nilchan *et al.,* 2024). Antigenic regions around 300–320 aa and 380–390 aa have been consistently highlighted as epitope clusters that elicit subtype-specific neutralising antibodies against the dengue virus (Lin *et al.,* 2015), suggesting the potential for specific immunological applications, including the development of diagnostic antigens for serotype-specific DENV detection.

This study demonstrates that IPTG-induced expression at 30°C provides optimal expression of recombinant EDIII proteins in E. coli BL21 (DE3) cells, with peak expression observed 4 to 6 hours post-induction (Fig. 9). In contrast, IPTG-induced expression at standard bacterial growth temperature (37°C) showed relatively lower E. coli growth density and expression levels (Fig. 9). Reduced induction temperature likely slows down the rate of protein synthesis, thereby facilitating proper folding of the target protein and improving overall expression efficiency. Several studies report that lowering induction temperature (20-30°C) in E. coli expression systems enhances solubility of recombinant proteins by slowing translation rates and allowing proper folding, thereby reducing inclusion body formation and improving yield of functional proteins (San-Miguel *et al.,* 2013; Z.S. Zhang *et al.,* 2007). These findings suggest that it is possible to improve the expression of recombinant EDIII protein in E. coli expression systems by improving the physicochemical properties and optimising culture conditions.

5.0 Limitations

Several limitations of this study should be noted. First, the concentration of expressed recombinant D2EDIII proteins was not quantified, limiting the ability to optimise and scale up expression conditions. Second, immunological assays to confirm the biological function and specificity of the recombinant EDII proteins were not conducted due to a lack of the required laboratory reagents. Despite these limitations, this study underscores the usefulness of *in silico* design of target proteins and optimised culture conditions for improving the expression of difficult-to-express proteins such as envelope domain III in the *Escherichia coli* BL21 (DE3) expression system.

6.0 Conclusion

The DENV-2 EDIII protein was successfully designed, codon-optimised and characterised using computational tools for recombinant expression in the *Escherichia coli BL21* (DE3) system. The EDIII construct demonstrated good predicted solubility, antigenic potential, and efficient expression in E coli BL21 (DE3) host cells. The results of this study support the potential of the EDIII domain as a promising candidate antigen for the development of serotype-specific dengue diagnostic assays. Overall, these findings highlight the value of integrating computational protein design with optimised low-temperature bacterial expression to produce recombinant proteins suitable for the development of specific immunodiagnostics.

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9.0 Declaration of Conflicting Interests

The author declares no conflict of interest.

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