Iran. J. Biotechnol. April 2023;21(2): e3288 DOI: 10.30498/ijb.2023.330285.3288

Research Article

Response Surface Methodology to Optimize the Expression Efficiency of Recombinant Reteplase

Farhad Farzaneh[1*], Sako Mirzaie[1], Ehsan Dehnavi[2], Mojtaba Aghaeepoor[2], Shirin Farzaneh[3], Navid Pourzardosht[4], Saeed Khalili[5]

[1]Department of Biochemistry, Faculty of Science, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran.
[2]Gene Transfer Pioneers (GTP) Research Group, Shahid Beheshti University of Medical Sciences. Tehran, Iran.
[3]Pharmaceutical Science Research Centre, Tehran medical Science, Islamic Azad University, Tehran, Iran.
[4]Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran;
[5]Department of Biology Sciences, Shahid Rajaee Teacher Training University, Tehran, Iran

[*]*Corresponding author*: Farhad Farzaneh, Department of Biochemistry, Faculty of Science, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran. Tel/ Fax: +98- 8733288661, E-mail: z.hashemi@acecr.ac.ir

Received: 2022/03/05 ; **Accepted:** 2022/12/13

Background: Over expression of Reteplase enzyme has already been studies in the periplasmic space of *Escherichia coli* (*E. coli*). However, the role different factors in its expresssin rate remained to be elucidated.

Objectives: Optical cell density (OD), IPTG concentration, and expression time are highly effective in the protein expression rates. Therefore, we aimed to determine the optimum levels of these factors for reteplase expression using response surface methodology (RSM).

Materials and Methods: The pET21b plasmid was used to sub-clone the designed reteplase gene. Then, the gene was transformed into *E. coli* BL21 strain. Induction of expression was done by IPTG and analyzed by the SDS page. experiments were designed using the RMS, while the effects of different conditions were evaluated using the Real time-PCR.

Results: Sequence optimization removed all undesirable sequences of the designed gene. Transformation into *E. coli* BL21 was confirmed with an 1152 bp band on the agarose gel. A 39 kDa expression band on the SDS gel confirmed the gene expression. Performing 20 RSM-designed experiments, the optimum levels for IPTG concentration and OD were determined as 0.34mM and 5.6, respectively. Moreover, the optimum level of expression time was demonstrated to be 11.91 hours.The accuracy of the regression model for reteplase overexpression was confirmed by an F-value equal to 25.31 and a meager probability value [(Prob > F) < 0.0001]. The real-time-PCR results indicated that the performed calculations were highly accurate.

Conclusion: The obtained results indicate that IPTG concentration, OD, and expression time are significantly involved in the augmentation of recombinant reteplase expression. To the best of our knowledge, this is the first study to assess the combined effect of these factors on reteplase expression. Further RSM-based experiments would bring about new insights regarding the best conditions for reteplase expression.

Keywords: Gene expression, Protein, Reteplase, Tissue plasminogen activator

1. Background

The blood clots (in the blood vessels) could lead to consequences like blood dysfunction and death due to their potential to trigger heart and brain strokes and pulmonary embolisms. These problems have become more prevalent due to the changes in the lifestyle of humans around the world (1, 2). Since blood clots remain to be a growing health issue, taking more efficient approaches seems to be imperative to prevent their incidence. In general, the fibrinolytic system is

***Copyright** © 2023 The Author(s); Published by National Institute of Genetic Engineering and Biotechnology. This is an open access article, distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses /by-nc/4.0/) which permits others to copy and redistribute material just in noncommercial usages, provided the original work is properly cited.*

responsible for the prevention of blood clots being speeded in the bloodstream. It also acts against the blood coagulation system (3, 4). The conversion of plasminogen pre-enzyme to plasmin is the critical process to initiate the fibrinolytic system. The tissue plasminogen activator (t-PA: a component of the serine protease enzymes) is the critical enzyme that converts plasminogen to plasmin. This property of the t-PA makes it and its derivatives considered amenable drugs of thrombolytic therapy to treat thrombotic disorders especially acute myocardial infarction. The t-PA is secreted into the bloodstream by endothelial cells that line various parts of the vascular system. The t-PA is a glycoprotein, which is comprised of 527 amino acids, and its molecular weight is reported to be 72 kD. This enzyme is composed of 5 structural domains, including the serine protease domain at the carboxyl terminus of the protein, the loop finger domain at the amino terminus of the protein, the kringle2 domain, the growth factor domain, and the kringle1 domain (5, 6). This enzyme is usually inactivated due to its interaction with an inhibitor molecule known as the Plasminogen Activator inhibitor-1 (7). The activation could occur in response to various stimuli, such as blockage of infertility and bradykinin (8). Despite its therapeutic significance, the recombinant production of t-PA is limited by its glycosylated nature. Production of glycosylated t-PA is costly, and the details of its production are not available. This limitation has directed the studies towards producing active and non-glycosylated derivatives of t-PA. Amongst, reteplase is a short and non-glycosylated version of t-PA, which is made by mutating the Alteplase (9). The Alteplase is a type of tissue plasminogen that dissolves blood clots in the cerebrovascular aorta. It has been employed to treat patients with acute stroke and is considered a thrombolytic agent. Alteplase converts the fibrin bond plasminogen to plasmin, which in turn causes fibrinolysis and dissolution of the clot. This enzyme has a limited effect on free and circulating plasminogen. Therefore, Alteplase could be called a fibrin-specific agent. The third-generation fibrinolytic agents like reteplase could address this limitation. This enzyme shares 355 amino acids with t-PA, which only includes the kringle2 and serine proteases domains. The lack of three other t-PA domains has reduced the ability of Reteplase to transplant on liver receptors and increased its half-life. Moreover, the effect of reteplase is reported to be increased on other enzymes, such Alteplase, Tenecteplase, Streptokinase, and Urokinase in plasma (10-12). Given these features, the study of Reteplase production and its therapeutic applications have garnered much attention (13, 14).

The bacterial hosts have already been widely used for recombinant production of Reteplase. The study by Sadeghi et al. has examined the use of tac promoters for Reteplase production (15). In another study, the *E. coli* TOP10 host was used for the Cloning and expression of the Reteplase enzyme (16). The cytoplasmic and periplasmic production of Reteplase has been investigated in *E. coli* BL21A host (17). The cloning and expression conditions of Reteplase in *E. coli* BL21A host have also been studied by the Zhang et al. group (18).

2. Objectives

Although various studies have been conducted for recombinant production of the reteplase enzyme in prokaryotic hosts, the extent of impact exerted by each contributing factor is not fully understood. In this regard, we have designed a study to investigate the effects of expression time, OD and IPTG concentration on improving the efficacy of reteplase expression. To obtain the optimal expression conditions, the RSM was employed (19-21). This method has already been used for optimization of various proteins (22, 23). In silico methods could be used to desing and engineer various proteins and their corresponding genes (24-27). A synthetic gene was designed in silico and sub-cloned into the PET21b plasmid. The *E. coli* BL21A host has been used to overexpress the designed gene. Changing the expression conditions in terms of expression time, OD and IPTG concentration based on the experiments designed by RSM has led to improved expression yields of the Interferon alpha-2b (28).

3. Materials and Methods

3.1. Materials, Culture Media, and Strains

Materials for PCR reactions and DNA extraction and recovery kits were obtained from the pioneer gene transfer company (Tehran-Iran). Restriction enzymes, T4 ligase, DNA marker, and protein marker were purchased from fermentase. All chemicals and salts were purchased from Merck (Germany). IPTG was prepared from Cinnagen (Tehran-Iran). The recombinant plasmid PET21b was purchased from the Danish Novozyme

Company. The DH5α and BL21 strains of *E. coli* were provided by the Institute of Pasteur of Iran. The BL21 strain was used for the cloning of the gene and the following protein expression, while the DH5α strain was used for long-term gene storage. Luria Bertani medium (LB) was used for bacterial growth. Ampicillin was purchased from Dana Pharmaceutical Company (Tabriz-Iran).

3.2. Codon Preference and Gene Synthesis

The designed gene is a part of the plasminogen activating a gene that encompasses 355 amino acids of its polypeptide. Reteplase protein sequence was used to prepare the synthetic gene (Accession No**.** KU053049.1) (hereafter the rt-PA gene). The gene sequence is 1065 base pairs in length, optimized according to the codon preference of *E. coli* strain BL21. The NCBI-related database at (http://www.kazusa.or.jp/codon) was used to optimize the gene sequence using the codon bias from *E. coli*. The secondary structure of the mRNA molecule, especially at the ribosome-binding site, was considered for gene design. The secondary structure of the mRNA was predicted using the mfold server at http://unafold.rna.albany.edu/?q=mfold. Moreover, the regions rich in AT and GC nucleotides that could inhibit protein expression were excluded. The prokaryotic ribosome binding sites, rho-independent transcription terminators, and the cleavage sites of restriction enzymes were avoided using the JCat server at http://www.jcat.de/. The gene sequence was supplemented by the sequence of Pel B signal sequence to ensure the periplasmic expression of the reteplase protein. The designed gene was ordered for syntheses within the pUC57 plasmid from the Shinegene Company (China).

3.3. Cloning of Synthetic Reteplase Gene into the E.coli BL21

All cloning methods were performed according to the standard protocols described by Sambrook *et al*., unless otherwise specified (29). The rt-PA gene (within the pUC57 plasmid) was introduced into the PET21b expression vector between the restriction sites of the BamH1 and Nde1 enzymes to create the PET21b-rt-PA construct. The standard enzymatic digestion (restriction enzymes: Nde1 and BamHI) and DNA ligation (enzyme: T4 DNA ligase) reactions were used to perform the cloning. The construct was transformed into the *E. coli* BL21 and DH5α cells by the $CaCl_2$ method. The transformation and cloning processes were screened by culturing the transformed bacteria on LB + 100 $mg.mL^{-1}$ ampicillin. Ultimately, T7 universal primers were used to confirm the cloning accuracy by colony-PCR method and gene sequencing.

3.4. Basic Reteplase Expression

A colony of *E. coli* BL21, which was confirmed to contain the PET21b-rt-PA recombinant plasmid, was picked for protein expression. The colony was cultured in 5 mL of ampicillin (40 µL of ampicillin in 50 mL of liquid LB medium) supplemented Luria-Bertani (LB) liquid medium at 37 °C in the incubator shaker. The following day, the specimens were cultivated in a 250 mL Erlenmeyer flask and placed in the incubator shaker at 37 °C and 250 rpm. The incubation continued to reach the OD of 0.4 to 0.6. At this stage, the isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) was added to the culture medium. The induction of protein expression was continued for 3 hours.

3.5. Protein Extraction and Preparation

To analyze the protein expression product, the cells were collected by centrifuging at 4500 g for 10 min at 4 °C. The resulting supernatant and precipitate were placed in two 2 mL vials. Since the gene is designed to accumulate the protein within the periplasmic space, a method previously developed by Khodabakhsh *et al*. was adapted to extract the protein from the periplasmic space with some adjustments (17). Following the bacterial cell sedimentation, the precipitate was homogenized on ice in a buffer of 30 mM Tris containing 20% sucrose and ethylenediamine tetraacetic acid (EDTA) (1 mM) at pH 8.0. Then, the sample was treated with lysozyme (0.25 $mg.mL^{-1}$) for 30 minutes on ice. The resulting solution was centrifuged for 50 minutes at 10000 g at 4 °C. The precipitate was re-suspended by 30 mL of another buffer containing 20 $mmoL.L^{-1}$ of EDTA, 0.1 $moL.L^{-1}$ of Tris, and 2.5% v/v of Triton X-100. The solution was subjected to another round of centrifugation at 4 °C and 10000 g. The obtained pellet was re-suspended in another buffer containing 20 $mmoL.L^{-1}$ of EDTA and 0.1 $moL.L^{-1}$of Tris. The obtained preparation inclusion body was stored at -20 °C. To dissolve the obtained inclusion bodies, the prepared samples were incubated at room temperature for 15 hr using a buffer containing

10 mM of EDTA, 25 mM of Tris-HCl, 2–8 M of guanidine hydrochloride, and 50 mM of DTT at pH 8.5. A refolding buffer containing 10 mM of EDTA and 25 mM of Tris-HCl (pH 8), along with various additives concentrations, was employed to refold the extracted protein into its native-like structure. The employed additives included Gn-HCl (2–8 M), arginine (0–1 M), and reduced and oxidized forms of glutathione (GSH/GSSG, at 10:1 ratios of reduced to oxidized glutathione). The samples were incubated in refolding buffer for 24 hr at 20 °C. Overnight dialysis (Sigma dialysis bag) at 4 °C against six moL.L^{-1} guanidine hydrochloride (pH 6) was used to isolate the refolded protein from the reducing agents and buffer components. Then, 50 µL of SDS sample buffer (2X) was added to protein samples and boiled for 5 min. A 15% (W/V) polyacrylamide gel was prepared to analyze the protein by the SDS-PAGE method. The gel staining and de-staining were done by 4 hr of incubation in standard Coomassie brilliant blue G-250 and 1 hr incubation in 45% methanol and 10% acetic acid solution, respectively. The amount of expressed protein was determined using the Bradford method. Bovine serum albumin (BSA) was used as the standard protein of the Bradford method.

3.6. Response Surface Methodology and Optimization of Expression Conditions

RSM technique is a statistical technique that can design the lowest number of experiments to unravel the most effective conditions for an experiment to reach the optimum yield. The Response Surface Methodology (RSM) and Central Composite Design (CCD) were employed to optimize the reteplase expression. The IPTG concentration, OD, and expression time were the cultivation conditions used for designing optimization experiments by Minitab 16 software (Minitab Inc., USA). After receiving the data, all the requirements obtained from the software were studied and analyzed. According to the calculations, 20 experiments were designed and carried out. The experiments contained six replicated center points. The experimental data were statistically analyzed by regression method:

$$Y = \beta 0 + \sum \beta i\ xi + \sum \beta iixii\ 2 + \sum \beta ijxi\ xj + \varepsilon$$

Where, Y is the predicted mRNA percentage of reteplase; β0 is a constant coefficient, βi the linear coefficient, Xi and Xj are input independent variable levels, βii is the quadratic coefficient, βij is the cross-product coefficient and ε is the residual error. Design-Expert software (version 8.0.7.1, StatEase Inc., Minneapolis, USA) was utilized to carry out the data analysis of the experimental design and the RSM. Experimental design and the RSM were performed by Design-Expert software (version 8.0.7.1, StatEase Inc., Minneapolis, USA).

3.7. Qualitative Evaluation of Reteplase Expression by Real Time-PCR and ΔΔCt Method

To assess the expression levels of reteplase in different experiments, the real-time PCR method was employed. In this regard, the total RNAs were extracted from the recombinant *E. coli* host cells in each experiment as test samples and the un-transformed cells as a negative control. The RNA samples were extracted using Trizol reagent (Life Technologies, USA) and following the instructions provided by the manufacturer. The obtained RNA specimen were assessed for their quantity and quality using the Nanodrop device at 260/280 nm and the gel electrophoresis, respectively. Following the RNA quality assessment, the cDNA was synthesized using the Thermo Scientific cDNA synthesis kit. In the process of cDNA synthesis, 50mg of total RNA (18 µL) and 1 µL of Random Hexamer primers were used to do the reverse transcription. The volumes of the reactions were set to contain 20 µL of RNase-free water. Following the cDNA preparation, SYBR Green PCR Master Mix (life technology) was used to perform the Real Time-PCR. The reactions were prepared according to the instructions provided by the supplier. This method would help to examine the levels of expression and confirm the optimization of protein expression exerted by the RSM method. Specific primers were designed using Oligo 7 primer analysis software, and 16sRNA primers (sense 5- CTACGGGAGGCAGCAGTGG -3 and antisense 5-TATTACCGCGGCTGCTGGC -3) were used as an internal control gene to determine the amount of mRNA .All specimens were assessed in two replicates, and their average value was recorded for each sample. The amplification cycles were set to have the annealing step at 60° for 1 min. The melting curve program was set to 66-99° with a continuous fluorescence measurement and a heating rate of 0.1°/s. A dissociation curve was also plotted to determine the specificity of amplification products. The variation in the gene expression level was determined using the $2^{-\Delta\Delta Ct}$ method.

4. Results

4.1. Gene Design and Optimization

The protein sequence of the Reteplase was used to be reverse transcribed into the rt-PA gene. The rt-PA gene was optimized according to the codon preference of the *E. coli* strain BL21. All undesirable sequences like AT and GC-rich regions, rho-independent transcription terminators, prokaryotic ribosome binding sites, and the cleavage sites of restriction enzymes were removed from the gene sequence (30). Moreover, there were no cryptic splicing sites, internal chi sites, ribosomal binding sites and repeat sequences within the optimized sequence. There was no highly stable secondary structure at the ribosome binding site of the rt-PA mRNA. This ensures that the translation from the mRNA can be done without inhibition. Highly stable secondary structures, especially at the ribosome binding site, could halt the translation machinery from proceeding along the mRNA molecule.

4.2. Construction of PET21b-rt-PA Recombinant Plasmid

The growth of the transformed bacteria on LB + 100 mg.mL^{-1} ampicillin indicates that the bacteria have been transformed with the PET21b, a plasmid that contains the resistance gene. The results of the colony-PCR method and gene sequencing showed that the rt-PA gene was successfully inserted into the PET21b expression vector between the restriction sites of the BamH1 and Nde1 enzymes (**Fig. 1**). The colony-PCR reactions have resulted in a 1152bp band, which indicates the correct insertion of the gene within the expression vector (**Fig. 1**). The sequence alignment between the sequenced gene and the designed gene revealed that the gene is sub-cloned without any sequence variations.

4.3. Reteplase Protein Expression

The results of the SDS-PAGE method revealed a 39 kDa protein band in the transformed samples, which was absent in the negative control sample (**Fig. 2**). Given that the calculated molecular weight for Reteplase is 39 kDa, the observed 39-kDa band could be deemed as rt-PA gene overexpression by the PET21b-rt-PA recombinant plasmid.

4.4. RSM Design

Five levels were produced for experimental ranges of each variable (-α, -1, 0, +1, +α) (**Table 1**). Then, to

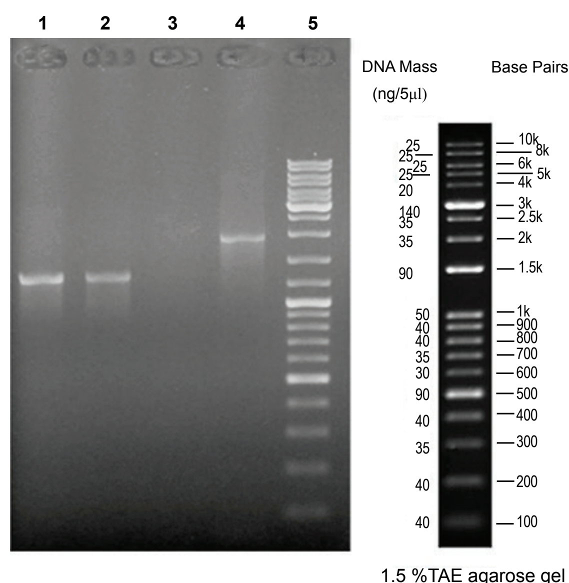


Figure 1. Colony-PCR analysis of transformants indicated that the synthetic reteplase gene was successfully cloned. Lanes 1-2 are colony-PCR on transformed colonies of *E. coli*, Lane 3 as a negative control, Lane 4 is pUC57-Asparginase plasmid as a positive control. Lane 5 is a DNA marker.

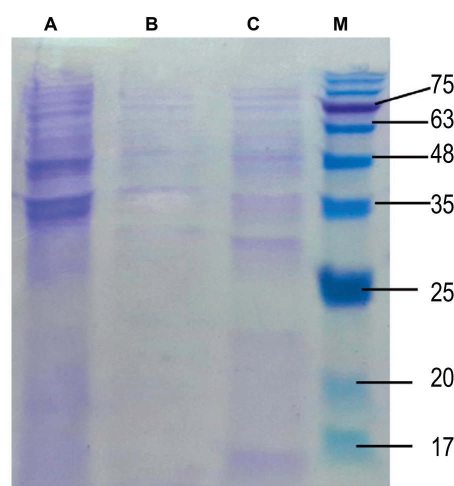


Figure 2. SDS-PAGE analysis of recombinant reteplase. Lane M is the protein molecular marker. Lane A-C are samples expression on SDS-PAGE gel. Lane A is the principal of expression, reteplase gene, lane B is the asparaginase gene as positive control, and lane C is Ecoli BL 21 –PET21 as negative control. The molecular weight of reteplase is 39KD.

Table 1. Assessed factors in Response Surface Methodology for expression optimization of recombinant reteplase, along with the values obtained from Real time-PCR and RSM in the dominant of measured and predicted response with transcription level of reteplase.

Run	Run Order	PtType	Blocks	Factor A IPTG (Mm)	Factor A IPTG (Mm)	Factor B Expression time (Hrs)	Factor B Expression time (Hrs)	Factor C Cell density	Factor C Cell density	Actual	Predicted
1	1	1	1	-1	0.15	-1	3	-1	1.4	80.5	76.5410
2	2	1	1	1	0.4	-1	3	-1	1.4	75.9	73.7039
3	3	1	1	-1	0.15	1	10	-1	1.4	64.8	73.7039
4	4	1	1	1	0.4	1	10	-1	1.4	61.2	61.7180
5	5	1	1	-1	0.15	-1	3	1	4.6	70.4	60.3809
6	6	1	1	1	0.4	-1	3	1	4.6	64.7	66.3999
7	7	1	1	-1	0.15	1	10	1	4.6	62.7	63.0628
8	8	1	1	1	0.4	1	10	1	4.6	59.0	60.0769
9	9	-1	1	-1.68	0.065	0	6.5	0	3	71.5	58.2398
10	10	-1	1	1.68	0.485	0	6.5	0	3	72.2	77.1654
11	11	-1	1	0	0.275	-1.68	0.62	0	3	69.3	73.2349
12	12	-1	1	0	0.275	1.68	12.38	0	3	55.4	73.9875
13	13	-1	1	0	0.275	0	6.5	-1.68	0.312	58.1	57.4673
14	14	-1	1	0	0.275	0	6.5	1.68	5.688	48.5	61.8550
15	15	0	1	0	0.275	0	6.5	0	3	82.2	51.5271
16	16	0	1	0	0.275	0	6.5	0	3	78.2	80.0081
17	17	0	1	0	0.275	0	6.5	0	3	83.2	80.0081
18	18	0	1	0	0.275	0	6.5	0	3	76.2	80.0081
19	19	0	1	0	0.275	0	6.5	0	3	83.2	80.0081
20	20	0	1	0	0.275	0	6.5	0	3	78.3	80.0081

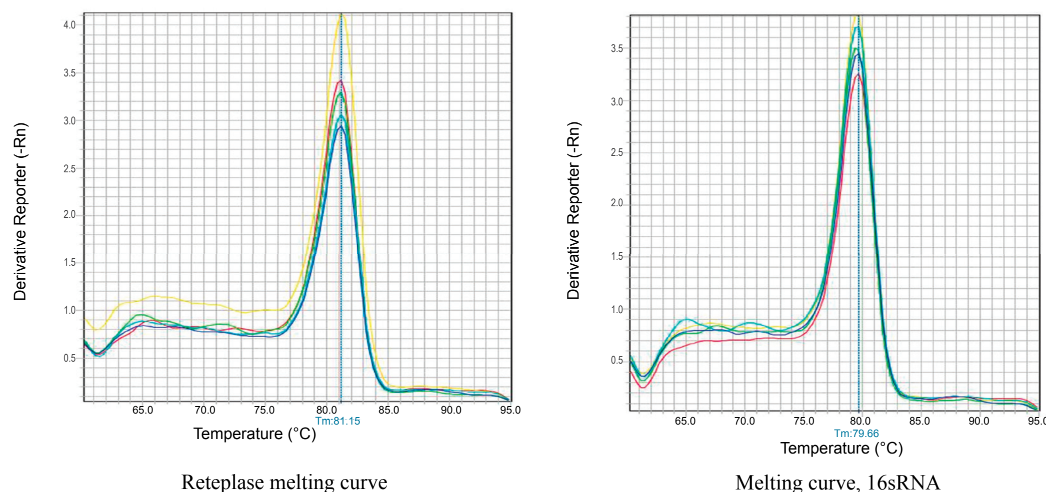
optimize the IPTG concentration, OD, and expression time (as selected parameters), 20 experiments were designed, which included six replications of the central points (**Table 1**). Some additional information is presented in **Table 1**. This information includes the assessed (by real-time method) percentage and the predicted amounts of the transcribed rt-PA mRNA under the Actual and Predicted columns. An equation obtained by regression method was used to calculate the predicted levels of mRNA expression as the function of IPTG concentration (A), OD (B), and expression time (C).

$$Y(\text{reteplase expressed mRNA Percentage}) = 80.0081 - (1.16855\times A) - (4.9115\times B1) - (3.07052\times C1) - (1.69987\times A1\times A1) - (5.04899\times B1\times B1) - (8.24382\times C1\times C1) + (0.375\times A1\times B1) - (0.125\times A1\times C1) + (2.125\times B1\times C1)$$

The results of the Real-time PCR technique were used to determine the changes in expression in each experimental condition. The specific forward 5-ATTCTGAGCGCAGCACATTG-3 and reverse 5-TCGTCGTCAAACTCTTTATG-3 were used to amplify the samples from each designed experiment. To assess the specificity of the amplification products, dissociation curves were drawn (**Fig. 3**). The $2^{-\Delta\Delta Ct}$ method was used for the evaluation of the relative changes in the levels of gene expression in recombinant reteplase. In **Table 1**, the values obtained by the Real-time PCR were considered actual values. The values of all columns (except for the two columns of Actual and Predicted) were originally proposed by the Mini Tab software to choose the experimental conditions and their implementation.

4.5. Model Validation and Experimental Confirmation

The statistical significance of the obtained equation for the response surface quadratic model was confirmed by performing an F-test and the analysis of variance



Reteplase melting curve

Melting curve, 16sRNA

Figure 3. The melting curves of Real time-PCR for recombinant reteplase and 16SRNA.

(ANOVA). The data about ANOVA analysis are presented in **Table 2**. The R^2 coefficient was stated to check the fitting of the model. The closer R^2 values to one show the forceful model and the superior response prediction. The predicted values are obtained from the independent variables in the CCD model, and the actual values are obtained for a specific run. The R^2 value was estimated to be 0.96, accordingly our results reveal that the regression model for reteplase overexpression is proper to the experimental values (**Fig. 4**). The accuracy of the model was confirmed by a Model F-value equal to 25.31 and a very low probability value [(Prob > F) < 0.0001]. The probability for the Model F-value to be a consequence of noise was as low as 0.01%. Any Model term with a "p-value of Prob > F" less than 0.0001 was considered significant. Given these circumstances, B, C, A2, B2 and C2 are the considerable model terms. In the case of too many insignificant model terms (except for the terms required to support hierarchy), the model could be improved by reduction. Since the R^2 value of the model was calculated to be 0.9043, it could be deduced that the obtained regression model for reteplase overexpression matches the experimental values (**Fig. 5**). Higher R^2 values (closer to 1) are associated with more robust models and a better ability to predict the responses. The independent variables in the CCD model were used to obtain the predicted values, and the actual values result from the specific run. Each designed experiment was empirically tested, and a graph generally called the optimal curves of RSM factors was plotted for them. The 3D surface plots (**Fig. 6**) were drawn for each factor to predict the effects of variation on the mRNA expression level and their optimum amounts. The maximum experimental (95.73) and predicted (93.6363) reteplase activity on the mRNA expression level was highly concordant (**Fig. 7**). The optimum levels for IPTG concentration, OD, and expression time were determined to be 0.34 mM, 5.6, and 11.91 Hours, respectively.

5. Discussion

Various thrombolytic drugs are prescribed after stroke diagnosis (31, 32). Despite their benefits in the treatment, each thrombolytic drug is associated with some disadvantages (33). A short half-life in blood plasma is one of these disadvantages, which reduces their effectiveness. Given these circumstances, the production of new generations of thrombolytic drugs seems to be imminently required. Reteplase is among these new generations of thrombolytic drugs, which are obtained by mutating alteplase (34). This enzyme has a distinct structure with high stability for prolonged exposure to blood plasma and high potency to degrade blood clots. The reteplase enzyme has already been expressed in the periplasmic space of *E. coli* (17). However, the effects of each variable on the optimization of the expression yield remained to be elucidated. In the present study, we have launched an

Table 2. Analysis of variance (ANOVA) for response surface quadratic model for reteplase production.

Source	Sum of square	F value	Prob>
Model	2127.47	2.83	0.0601
A-IPTG mM	21.66	0.26	0.6214
B-expression time (h)	319.75	3.83	0.0787
C-cell density (OD600)	480.63	5.76	0.0373
AB	11.52	0.14	0.7179
AC	11.05	0.13	0.7235
BC	11.04	0.13	0.7235
A2	46.88	0.56	0.4707
B2	7.20	0.086	0.7750
C2	1217.87	14.60	0.0034

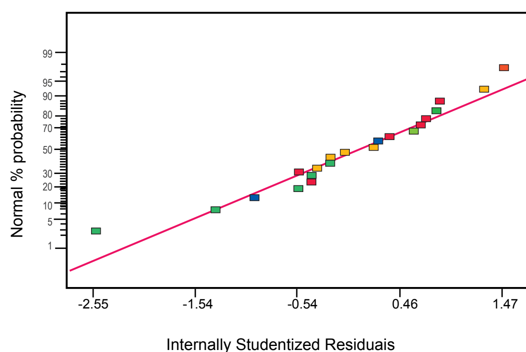


Figure 4. Predicted response versus actual value.

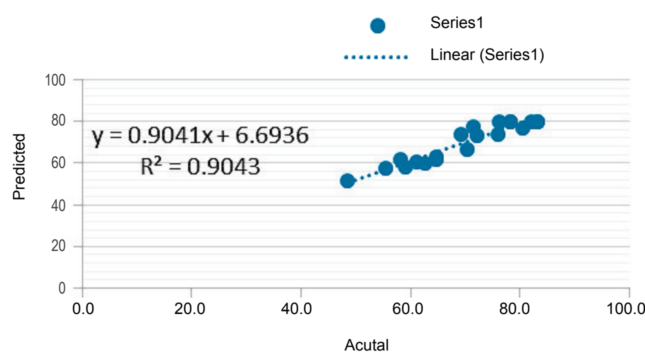


Figure 5. A normal plot of residuals and predicted response versus actual value.

RSM design to investigate the effects of changes in IPTG concentration, OD, and expression time on the periplasmic expression yield of the reteplase enzyme. Since the codon optimization of the designed gene has removed all undesirable sequences, the observed strong expression band within the expected size ensures the correct sub-cloning of the synthesized gene. The choice of an expression system to produce a recombinant protein from a large mammal depends on many factors, such as intracellular and extracellular expression, cell growth characteristics, and post-translational changes (35, 36). All of these factors can play a role in the expression of the recombinant gene. A T7 promoter is exploited within the PET21b vector, which could be one of the factors with a significant effect on the expression levels (37, 38). The additional protein band in the sub-cloned strain could be the indication of correct T7 promoter function in the expression of the recombinant gene.

The optimum condition for the expression of recombinant proteins could be affected by different factors. It should be noted that the overexpression of a protein by a plasmid DNA exerts a metabolic burden, which could hamper the specific growth rate and reduce the biomass content. These changes could end with the instability of the plasmid DNA. The upper limit of the specific growth rate is determined by the onset of glucose overflow metabolism and acetate formation. These factors are destructive to recombinant protein expression. Given these circumstances, finding optimum expression conditions seems to be highly required. In this regard, RSM is one of the most accurate multivariate analysis methods capable of simultaneous changing of several parameters (39-41). To determine the optimized conditions for periplasmic expression of the r-PA, the IPTG concentration, OD, and expression time were selected as variables for RSM. According to reports, the most crucial production factors to be tuned

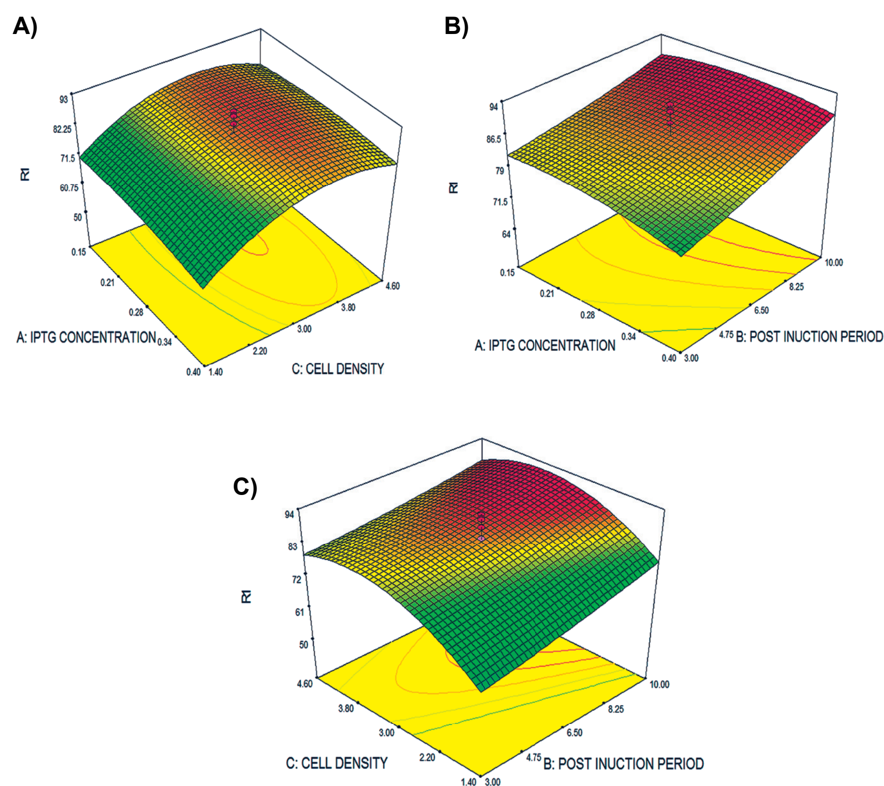


Figure 6. Three-dimensional response surface diagrams of the most efficient optimal conditions of expression. The effect of two variables are shown while the other variale is held at 0. The plots are showing the effect of time and IPTG concentration **A**), IPTG concentration and OD amount **B**), and OD amount and duration of expression **C**) on reteplase expression.

for high-yield recombinant protein expression are IPTG concentration, post-induction time, and cell density. Lowering the concentration of the induction agent is a way of slowing down the transcription rate. Additionally, increasing the production of a soluble protein can be achieved by reducing the IPTG concentration. Even though 0.1 to 1.0 mM IPTG concentrations are the most typical range for protein induction, solubility can be affected by reductions to even lower concentrations (42). Moreover, growing the culture to higher densities can enhance recombinant protein overexpression in different ways. This can be accomplished by altering a few factors, such as the medium composition, and by vigorously shaking to improve aeration (43) ; therefore, finding the optimum OD for induction would help get higher expression rates. In addition, It should be noted that more prolonged post-induction incubation of the expression system does not always lead to higher expression rates. After prolonged cultivation of the expression system, proteases are believed to be responsible for the degradation of heterologously produced proteins (44). Thus, finding the optimum duration of expression would help to avoid degradation issues and lower the expenses. Varying one parameter at a time whilst keeping the others constant is the conventional approach for optimized protein expression. However, this approach requires a high number of experiments, which could lead to misinterpretation of results in the case of interactions between different variables. RSM is an alternative approach to overcome these limitations.

The investigations conducted by the Mini Tab software (to perform RSM) presented the modes with the best conditions for expression. The factors that were evaluated in it are significant in reaching the most efficient and optimal expression conditions. It should be noted that at a 99% (p 0.05) level of confidence, analysis of variance (ANOVA) results for the RSM show that

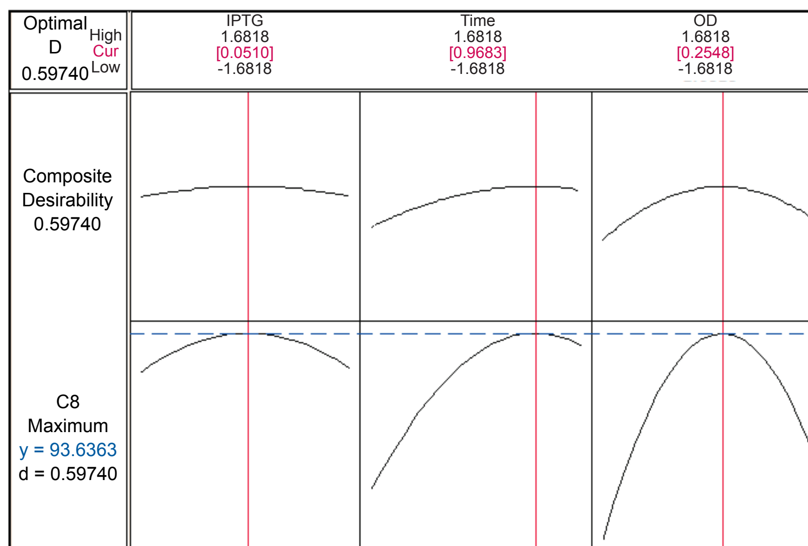


Figure 7. Optimization plot for highest reteplase expression.

the regression is statistically significant. It indicates that model terms are substantial if "Prob > F" values are less than 0.0500. Values above 0.1000, however, are not noteworthy. B, C, A2, B2 and C2 are significant model terms in this scenario. All other interactions are, therefore, not important, and these independent components do not interact effectively. The model's F-value of 25.31 shows that it is significant. A "Model FValue" this big might happen because of noise just 0.01% of the time. Two-dimensional contour plots show the evaluated expression conditions at their optimal levels. Given that they are all within predetermined ranges, the optimal values are acceptable. Arriving at optimum levels of IPTG is essential due to its high cost and potential for toxicity. The IPTG is responsible for the reduced growth rate and production of bacterial proteases capable of degrading heterologous proteins at high concentrations. Therefore, finding optimized levels of IPTG could essentially help the recombinant expression. The optimum level of IPTG was previously determined to be in a range of 0.005 to 5 mM. Larentis *et al*. have demonstrated that using 10-fold lower levels of IPTG could lead to better expression results (45). This amount is in concordance with the results of previous studies. The maximum experimental (95.73) and predicted (93.6363) r-PA activity on the mRNA expression level was highly concordant, which indicates the accuracy of performed predictions.

It could be concluded that RSM is capable of optimizing the expression condition for r-PA. These conditions could be used in more cost-effective industrial production of r-PA. Although our study improved the expression yield of the r-PA, further studies on the optimization of other parameters could lead to better expression yields.

Funding

No funds, grants, or other support was received.

Competing Interests

None

Ethics approval

Not applicable

Acknowledgments

The authors wish to thank the Islamic Azad University for their support to conduct this study.

References

1. Abed MA, Ali RMA, Ras MMA, Hamdallah FO, Khalil AA, Moser DK. Symptoms of acute myocardial infarction: A correlational study of the discrepancy between patients' expectations and experiences. *Int J Nurs Stud*. 2015;**52**(10):1591-1599.doi: 10.1016/j.ijnurstu.2015.06.003.
2. De Luca G, Suryapranata H, Ottervanger JP, Antman EM. Time delay to treatment and mortality in primary angioplasty for acute myocardial infarction: every minute of delay counts. *Circulation*.

2004;**109**(10):12235. doi:10.1161/01.CIR.0000121424.76486.20.
3. Chapin JC, Hajjar KA. Fibrinolysis and the control of blood coagulation. *Blood Rev*. 2015;**29**(1):17-24. doi: 10.1016/j.blre.2014.09.003.
4. Elnager A, Abdullah WZ, Hassan R, Idris Z, Wan Arfah N, Sulaiman S, *et al*. *In vitro* whole blood clot lysis for fibrinolytic activity study using D-dimer and confocal microscopy. *Adv Hematol*. 2014;**2014**:1-8. doi: 10.2255/2014/814684.
5. Manosroi J, Tayapiwatana C, Götz F, Werner RG, Manosroi A. Secretion of active recombinant human tissue plasminogen activator derivatives in Escherichia coli. *Appl Environ Microbiol*. 2001;**67**(6):2657-2664. doi: 10.1128/AEM.67.6.2657-2664.2001.
6. Papadopoulos SM, Chandler WF, Salamat MS, Topol EJ, Sackellares JC. Recombinant human tissue-type plasminogen activator therapy in acute thromboembolic stroke. *J Neurosurg*. 1987;**67**(3):394-398. doi: 10.3171/jns.1987.67.3.0394
7. Yasar Yildiz S, Kuru P, Toksoy Oner E, Agirbasli M. Functional stability of plasminogen activator inhibitor-1. *Sci World J*. 2014;**2014**:858293. doi: 10.1155/2014/858293.
8. Golias C, Charalabopoulos A, Stagikas D, Charalabopoulos K, Batistatou A. The kinin system-bradykinin: biological effects and clinical implications. Multiple role of the kinin system-bradykinin. *Hippokratia*. 2007;**11**(3):124-128.
9. Wooster MB, Luzier AB. Reteplase: a new thrombolytic for the treatment of acute myocardial infarction. *Ann Pharmacother*. 1999;**33**(3):318-324.doi: 10.1345/aph.18006.
10. Semba CP, Murphy TP, Bakal CW, Calis KA, Matalon TA. Thrombolytic therapy with use of alteplase (rt-PA) in peripheral arterial occlusive disease: review of the clinical literature. *J Vasc Interv Radiol*. 2000;**11**(2 Pt 1):149-161.doi: 10.1016/s1051-0443(07)61459-6.
11. Martin U, Kaufmann B, Neugebauer G. Current clinical use of reteplase for thrombolysis. *Clin Pharmacokinet*. 1999;**36**(4):265-276. doi: 10.2165/00003088-199936040-00002.
12. Sugg RM, Noser E, Shaltoni H, Gonzales N, Campbell M, Weir R, *et al*. Intra-arterial reteplase compared to urokinase for thrombolytic recanalization in acute ischemic stroke. *Am J Neuroradiol*. 2006;**27**(4):769-773.
13. Simpson D, Siddiqui MAA, Scott LJ, Hilleman DE. Spotlight on reteplase in thrombotic occlusive disorders. *BioDrugs*. 2007;**21**(1):65-68.doi: 10.2165/00063030-200721010-00008.
14. Eesa M, Schumacher HC, Higashida RT, Meyers PM. Advances in revascularization for acute ischemic stroke treatment: an update. *Expert Rev Neurother*. 2011;**11**(8):1125-1139.doi: 10.1586/ern.11.102.
15. Aghaabdollahian S, Rabbani M, Ghaedi K, Sadeghi HMM. Molecular cloning of Reteplase and its expression in *E. coli* using tac promoter. *Adv Biomed Res*. 2014;**3**:190-195. doi: 10.4103/2277-9175.140622.
16. Khodabakhsh F, Dehghani Z, Zia MF, Rabbani M, Sadeghi HMM. Cloning and expression of functional reteplase in Escherichia coli top10. *Avicenna J Med Biotechnol*. 2013;**5**(3): 168-175.
17. Khodabakhsh F, Zia MF, Moazen F, Rabbani M, Sadeghi HMM. Comparison of the cytoplasmic and periplasmic production of reteplase in *Escherichia coli*. *Prep Biochem Biotechnol*. 2013;**43**(7):613-623.doi: 10.1080/10826068.2013.764896.
18. Zhao Y, Ge W, Kong Y, Zhang C. Cloning, expression, and renaturation studies of reteplase. *J Microbiol Biotechnol*. 2003; **13**(6):989-992.
19. Aghaeepoor M, Akbarzadeh A, Kobarfard F, Shabani AA, Dehnavi E, Aval SJ, *et al*. Optimization and high level production of recombinant synthetic Streptokinase in *E. coli* using Response Surface Methodology. I*ran J Pharm Res*. 2019;**18**(2):961-973.doi: 10.22037/ijpr.2019.1100636.
20. Akbarzadeh A, Dehnavi E, Aghaeepoor M, Amani J. Optimization of recombinant expression of synthetic bacterial phytase in Pichia pastoris using response surface methodology. *Jundishapur J Microbiol*. 2015;**8**(12): e27553. doi: 10.5812/ijm.27553.
21. Aghaeepoor M, Kobarfard F, Eidgahi MRA, Shabani A, Dehnavi E, Ahmadi M, *et al*. Optimization of culture media for extracellular expression of streptokinase in *Escherichia coli* using response surface methodology in combination with Plackett-Burman Design. *IJPR*. 2017;**16**(11): 2567-2576.doi: 10.4314/TJPR.V1611.1.
22. Ashrit P, Sadanandan B, Kyathsandra Natraj L, Shetty K, Vaniyamparambath V, Raghu AV. A microplate-based Response Surface Methodology model for growth optimization and biofilm formation on polystyrene polymeric material in a Candida albicans and *Escherichia coli* co-culture. *Polym Adv Technol*. 2022; **33**(9): 2872- 2885.doi:10.1002/pat.5753.
23. Khalilvand AB, Aminzadeh S, Sanati MH, Mahboudi F. Media optimization for SHuffle T7 Escherichia coli expressing SUMO-Lispro proinsulin by response surface methodology. *BMC Biotechnol*. 2022;**22**(1):1. doi: 10.1186/s12896-021-00732-4.
24. Hashemi ZS, Zarei M, Fath MK, Ganji M, Farahani MS, Afsharnouri F, *et al*. In silico Approaches for the Design and Optimization of Interfering Peptides Against Protein–Protein Interactions. *Front Mol Biosci*. 2021;**8**:669431. doi: 10.3389/fmolb.2021.669431.
25. Khalili S, Rasaee MJ, Mousavi SL, Amani J, Jahangiri A, Borna H. In silico prediction and *in vitro* verification of a novel multi-epitope antigen for HBV detection. *Mol Genet Microbiol Virol*. 2017;**32**(4):230-240. doi: 10.3103/S0891416817040097
26. Rahbar MR, Zarei M, Jahangiri A, Khalili S, Nezafat N, Negahdaripour M, *et al*. Trimeric autotransporter adhesins in Acinetobacter baumannii, coincidental evolution at work. *Infect Genet Evol*. 2019;**71**:116-127. doi: 10.1016/j.meegid.2019.03.023.
27. Khalili S, Mohammadpour H, Barough MS, Kohaei P. ILP-2 modeling and virtual screening of an FDA-approved library: a possible anticancer therapy. *Turk J Me Sci*. 2016;**46**(4):1135-1143. doi: 10.3906/sag-1503-2.
28. Samarin ZE, Abolghasemi S, Dehnavi E, Akbarzadeh A, Hadian A, Khodab M, *et al*. Response Surface Optimization of the Expression Conditions for Synthetic Human Interferon alpha-2b Gene in *E. coli*. *Indian J Pharm Sci*. 2018;**80**(3):470-479. doi: 10.4172/pharmaceutical-sciences.1000380
29. Sambrook H. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY. Molecular cloning–Laboratory manuals. 1989. ISBN: 978-1-936113-41-5
30. Abe H, Abo T, Aiba H. Regulation of intrinsic terminator by translation in Escherichia coli: transcription termination at a distance downstream. *Genes Cells*. 1999;**4**(2):87-97.doi: 10.1046/j.1365-2443.1999.00246.x.
31. Balami JS, Chen R, Sutherland BA, Buchan AM. Thrombolytic agents for acute ischaemic stroke treatment: the past, present and future. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders). CNS Neurol. *Disord Drug Targets*. 2013;**12**(2):145-154. doi: 10.2174/18715273113129990057.
32. Bivard A, Lin L, Parsonsb MW. Review of stroke thrombolytics.

J Stroke. 2013;**15**(2):90-98. doi: 10.5853/ios. 2013.15.2.90.
33. Nascimento BR, de Sousa MR, Demarqui FN, Ribeiro ALP. Risks and benefits of thrombolytic, antiplatelet, and anticoagulant therapies for st segment elevation myocardial infarction: Systematic review. *ISRN Cardiol*. 2014;**2014**:1-11.doi: 10.1155/2014/416253.
34. GUSTO III A. A comparison of reteplase with alteplase for acute myocardial infarction: the global use of strategies to open occluded coronary arteries. *N Engl J Med*. 1997;**337**(16):1118-1123.doi: 10.1056/NEJM199710163371603.
35. Meyer S, Lorenz C, Baser B, Wördehoff M, Jäger V, van den Heuvel J. Multi-host expression system for recombinant production of challenging proteins. *Plos One*. 2013;**8**(7): e68674. doi: 10.1371/journal.pone.0068674.
36. Gao W, Yin J, Bao L, Wang Q, Hou S, Yue Y, *et al*. Engineering extracellular expression systems in *E. coli* based on transcriptome analysis and cell growth state. *ACS Synth Biol*. 2018;**7**(5):1291-1302. doi: 10.1021/acssynbio.7b00400.
37. Vahedi F, Khatami F, Es-haghi A. The stability of pET21+ PA, a pET derived plasmid, under different culture conditions. *World J Microbiol Biotechnol*. 2011;**27**(2):253-257. doi: 10. 1007/s11274-010-0452-1
38. Tabor S. Expression using the T7 RNA polymerase/promoter system. *Curr Protoc Mol Biol*. 1990;**11**:1621-1211.doi: 10.1002/ 0471142727.mb1602s11.
39. Breig SJM, Luti KJK. Response surface methodology: A review on its applications and challenges in microbial cultures. *Mater Today Proc*. 2021;**42**(5):2277-2284.doi: 10.1016/j.matpr. 2020.12.316.
40. Weremfo A, Abassah-Oppong S, Adulley F, Dabie K, Seidu-Larry S. Response surface methodology as a tool to optimize the extraction of bioactive compounds from plant sources. *J Sci Food Agric*. 2023;**103**(1):26-36. doi: 10.1002/jsfa.12121.
41. Chelladurai SJS, Murugan K, Ray AP, Upadhyaya M, Narasimharaj V, Gnanasekaran S. Optimization of process parameters using response surface methodology: A review. *Mater Today Proc*. 2021;**37**:1301-1304. doi: 10.1016/j.matpr.2020.06.466.
42. Francis DM, Page R. Strategies to optimize protein expression in *E. coli*. *Curr Protoc Protein Sci*. 2010; **61**(5):1-29. doi: 10. 1002/0471140864.ps0524s61.
43. Blommel PG, Becker KJ, Duvnjak P, Fox BG. Enhanced bacterial protein expression during auto-induction obtained by alteration of lac repressor dosage and medium composition. *Biotechnol Prog*. 2007;**23**(3):585-598.doi: 10.1021/bp070011x.
44. Choi JH, Keum KC, Lee SY. Production of recombinant proteins by high cell density culture of *E. coli*. *Chem Eng Sci*. 2006;61(3): 876-885. doi: 10.1016/j.ces.2005.03.031.
45. Larentis AL, Argondizzo APC, dos Santos Esteves G, Jessouron E, Galler R, Medeiros MA. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *E. coli* and recombinant protein stability during long-term storage. *Protein Expr Purif*. 2011;**78**(1):38-47. doi: 10.1016/j.pep.2011.02.013.