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Research Article

Response Surface Methodology Approach to Optimize the Expression of Thioredoxin-MOG Fusion Protein

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Abstract

Background: The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) has been shown to generate experimental autoimmune encephalomyelitis (EAE), an animal model of MS. A considerable amount of MOG must be accessible for EAE induction. Here, for the first time, Response Surface Methodology-Box-Behnken (RSM-BBD) was employed to identify the ideal culture conditions for causing *Escherichia coli (E. coli)* BL21 to overproduce the Thioredoxin-MOG (Trx-MOG) fusion protein. The RSM method is a powerful, efficient, and reliable alternative to the One-Factor-At-A-Time (OFAT) method in optimizing process variables, allowing for a smaller number of experimental runs, investigating variable interaction, and being cheaper and less time-consuming.

Methods: Here, using the 29 experimental assays, the direct and indirect effects of factors including post-induction time, IPTGinducer concentration, pre-induction optical density, and post-induction temperature on the protein expression level content were evaluated.

Results: The proposed quadratic model demonstrated a significant effect of the two variables A (time) and C (temperature) on protein synthesis. An inducer concentration of 0.491 mM, the pre-induction optical density (OD_{600}) of 0.8, and a temperature of 23 °C for 23.878 hours were found to be the best growth conditions for high yield Trx-MOG synthesis. The optimum protein concentration was attained (163.96 µg/mL) and was within the range of (200.04 µg/mL), which was the value predicted.

Conclusion: The study concluded that RSM optimization effectively increased the production of Trx-MOG in *E. coli*, which could have the potential for large-scale fermentation.

Introduction

Multiple sclerosis (MS), a multifocal demyelinating disease of the central nervous system (CNS), causes the myelin sheath that surrounds axons to gradually deteriorate. It can exhibit a variety of pathological and clinical symptoms, which may indicate the participation of many pathogenic processes.[1] Although there is currently no cure for MS, there are a number of disease-modifying therapies (DMTs) that can be used to manage and decrease the disease's progression. Experimental autoimmune encephalomyelitis, or EAE, is an example of MS animal model often used to study DMTs. It is usually induced in rats by active immunization, and is considered one of the most reliable animal models for studying the mechanisms underlying human illness. During active induction, an emulsion containing neuro-antigen peptides like myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), or proteolipid protein (PLP), along with pertussis toxin (PTx) and complete freund's adjuvants (CFA), is administered to the animal.[2] A considerable amount of autoantigen such as MOG must be accessible for the immunization-based induction of EAE.[3,4] With the aim of increasing the yield of recombinant proteins produced in *E. coli*, numerous fusion tags have been described thus far. As previously mentioned, thioredoxin (Trx) is the ideal fusion tag for expressing a protein with disulfide links.[5] For instance, Trx was able to increase the Trx-EpEX scFv fusion protein's expression level to 22.8 and 22.3% of the total protein in Origami™ (DE3) and Rosetta™ (DE3), respectively.[6] As was already indicated, careful tuning of the culture conditions can also result in high levels of protein expression.[7] To optimize culture conditions using the one-factor-at-a-time (OFAT) approach, several trial-and-error experiments are required before an optimal result is obtained. The actual ideal circumstances for the optimum answer, though, might not be met. In addition, it is impossible to identify how the many factors interact with one another in this approach.[8] DoE's ability to get beyond the shortcomings of the OFAT method during optimization has two key advantages: identifying important elements and the interactions between them. This statistical strategy can lead to a more accurate forecast of the real optimum

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with fewer experiments.[9,10] Response surface methodology (RSM) is one of the several DoEs used for developing empirical models.[5] Numerous research utilized the RSM-Box-Behnken design to improve the protein production procedure, both upstream and downstream.[9] For example, in a study by Jafari *et al.*[11], the RSM-BBD approach was used to develop culture conditions that optimally generated anti-keratin scFv TS1-218, exhibiting a 21-fold increase. They assessed how pH, methanol concentration, and temperature affected the expression of proteins. Additionally, using the BBD-RSM approach, Emamipour *et al.*[12] effectively enhanced DsbA-IGF1 expression and purification.

This study used RSM-BBD methodology for the first time to identify an optimal cultivation condition to overproduce the Thioredoxin-MOG (Trx-MOG) fusion protein in *E. coli* BL21 (DE3), taking into account variables such as induction temperature, post-induction time, pre-induction optical density (OD_{600}), and IPTG concentration.

Methods

Experimental design

In this study, using the RSM-BBD methodology, a thorough investigation was carried out into how four independent factors (post-induction time, IPTG concentration, pre-induction optical density, and temperature) affected the recombinant protein's expression level (Table 1). A four-factor-three-level BBD with 5 RSM center point replicates and 24 factorial points resulted in a set of 29 trials (Table 2). The tests were designed and the data were analyzed utilizing the Design-Expert software (Version 7.0.0, Stat-Ease Inc., USA). Furthermore, for model validation, the optimum condition leading to the maximum amount of the recombinant protein was experimentally confirmed.[13]

Bacterial strains, plasmid and growth media

The cloning host for the creation of the plasmid was the *E. coli* strain (DH5). The *Escherichia coli* BL21 (DE3) purchased from Pasteur institute of Iran, I.R. Iran, was utilized as a host for the Trx- MOG development. The pET32a(+) expression vector (Pasteur institute of Iran, I.R. Iran) was used to clone gene encoding the N-terminal extracellular IgG V-like MOG domain. Thermo Fisher Scientific in the United States provided all of the restriction enzymes. The gel extraction kit (DNAbiotech, I.R. Iran) was used to separate DNA fragments from an agarose gel. The bacterial strains were grown in Luria-Bertani (LB) medium, which was made up of 10 g/L tryptone (Merck, Germany), 5 g/L yeast extract (Merck, Germany) and 10 g/L NaCl (Merck, Germany).

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Construction of pET32a(+)- MOG

The enzymatic digestion of the pGH-MOG vector was performed using *Nco*I and *Hind*III enzymes in Tango 2x buffer for 18 hours at 37 °C (the pGH-MOG vector was supplied by Generay Biotech Co. in China and contained the synthesized N-terminal extracellular domain of MOG gene (378bp) (GenBank accession no. NM_001008228) coupled with a C-terminal hexahistidine tag).[14] After digestion, MOG fragment were purified with the High Pure PCR Product Purification Kit (DNAbiotech, IRAN) based on the manufacturer's protocol. The recombinant expression vector (pET32a(+)-MOG) was created by inserting the purified gel-extracted MOG fragment into the *Nco*I/*Hind*III sites of pET32a(+) vector (Figure 1). Briefly, after combining the DNA fragments (insert and vector), DNA ligase buffer, and T4 DNA Ligase enzyme in a reaction mixture, the mixture was incubated at 4 °C for 16 hours. The reaction was then terminated by heat inactivation.[14] Competent *E. coli* (DH5) was transformed with the pET32a(+)-MOG plasmid. Briefly, after combining competent cells and the recombinant plasmid in a tube, it was incubated on ice for 30 minutes. The mixture was then shocked at 42 °C for a short period. The tube was transferred back to ice and the cells were allowed to recover for 5-10 minutes. After adding the medium to the transformed cells, they were incubated at the appropriate temperature for recovery and outgrowth. On LB plates

Table 1. Independent variables and their corresponding levels.

Symbols	Variables	Levels		
		-1	0	+1
A	Post-induction time (h)	6	15	24
B	Cell density (OD_{600}nm)	0.6	0.7	0.8
C	Post-induction temperature (°C)	23	30	37
D	IPTG concentration (mmol/L)	0.4	0.7	1

IPTG, Isopropyl-b-D-thiogalactopyranoside.

Table 2. The Box Behnken design with measured response.

Experiments	Temperature (A) (˚C)	OD (B) (600 nm)	Time (C) (h)	IPTG (D) (mM)	Trx-MOG production (µg)	
					Actual	Predicted
1	30	0.6	15	1	979.42	1077.28
2	30	0.6	6	0.7	915.10	815.96
3	23	0.6	15	0.7	1521.70	1375.90
4	30	0.6	15	0.4	900.96	932.68
5	37	0.6	15	0.7	752.910	897.47
6	30	0.6	24	0.7	916.06	886.86
7	37	0.8	15	0.7	952.67	879.30
8	30	0.7	6	1	463.20	338.93
9	23	0.7	6	0.7	396.19	496.78
10	30	0.7	24	0.4	832.73	845.71
11	30	0.7	6	0.4	570.39	454.64
12	37	0.7	6	0.7	404.050	560.20
13	23	0.7	24	0.7	1600.09	1429.71
14	37	0.7	24	0.7	524.23	409.41
15	30	0.7	24	1	580.95	730.00
16	30	0.8	6	0.7	395.21	477.62
17	30	0.8	24	0.7	1036.50	1188.86
18	30	0.8	15	1	844.75	798.80
19	30	0.8	15	0.4	1286.90	1174.82
20	30	0.7	15	0.7	484.02	682.70
21	30	0.7	15	0.7	698.66	682.70
22	30	0.7	15	0.7	678.08	682.70
23	23	0.8	15	0.7	1361.10	1357.73
24	37	0.7	15	0.4	766.81	696.23
25	37	0.7	15	1	622.47	580.52
26	23	0.7	15	0.4	920.96	1174.67
27	30	0.7	15	0.7	885.65	682.70
28	30	0.7	15	0.7	667.09	682.70
29	23	0.7	15	1	1093.70	1058.96

containing ampicillin (100 µg/mL), the transformed cells were cultured at 37 °C overnight.[14] A plasmid extraction kit (DNAbiotech, IRAN) was used to isolate plasmids from positive clones. The proper frame of the cloned fragment was verified by polymerase chain reaction (PCR) carried out by utilizing primers designed by Oligo7 Software (version 7.54, Molecular Biology Insights Inc., USA) (forward: 5′-GCCGCTGTGATCTTTTGCTTAAT-3′ and reverse: 5′-GGTTGGAGGTGTTGTATAATGCTG CTG-3′). Using a thermal cycler (Analytik Jena AG, Germany), amplification was done under the following temperature conditions: (1) initial DNA denaturation for 5 minutes at 95 °C; (2) 35 PCR cycles at 94 °C for 45 seconds, 53 °C for 40 seconds, and 72 °C for 1 minute; and (3) an extension period for 7 minutes at 72 °C.

Protein expression

The plasmid pET32a(+)-MOG was transformed into competent *E. coli* BL21 (DE3) cells for initial protein expression determination using the heat shock method, and a single colony of *E. coli* BL21 (DE3) containing pET32a(+)-MOG was cultivated for 20 h at 37 °C in Luria-Bertani broth containing ampicillin (100 µg/mL). From a 5 mL Luria–Bertani (LB) overnight, cultures were inoculated 10% by volume (v/v) into 30 mL of LB broth and incubated at 37 °C in a rotary shaker (200 rpm) till the OD_{600} attained 0.6-0.8. After that, 1 mmol/L of isopropyl-b-D-thiogalactopyranoside (IPTG, Sigma, Germany) was added. Cells were extracted by centrifugation (10000 g; 10 min at 4 °C) after 2, 4, 6, and 24 hours of induction and stored at -20 °C for future analysis. For optimization study, all the experiments were conducted in sterile 50 mL-falcon tube with 10 mL of LB medium (range of post-induction time: 6-24 hours, range of post-induction temperature: 23-37 °C, range of pre-induction optical density: 0.6-0.8, and range of the inducer concentration: 0.4-1 mmol/L). Cells

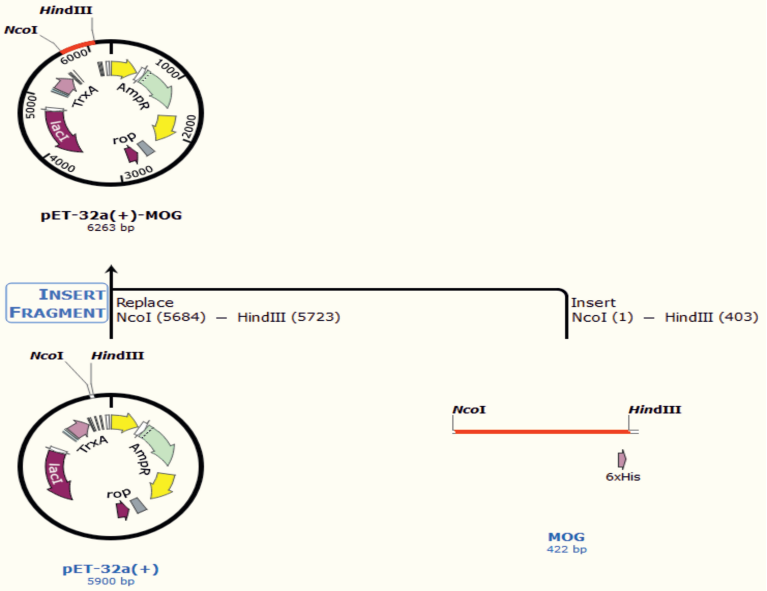

Figure 1. Schematic diagram of construction of the pET32a(+)-MOG vector. The MOG gene and its corresponding endonuclease sites were inserted into the NcoI and HindIII restriction sites located downstream of the T7 promoter region within the vector. Design of plasmid was carried out using a vector mapping software (SnapGene Version 3.2.1, available at snapgene.com).

were extracted by centrifuging the fermentation broth at 4 °C (10000 g for 10 minutes). The pellet was resuspended in lysis solution (5 mL) containing NaCl (50 mM), Tris (20 mM, pH 7.5), and glycerol (50%). After sonication (15 minutes (7/8-s on/off) at 300 W), the cells were centrifuged at 10000 g for 30 minutes at 4 °C.[13] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, USA) was utilized to determine the expression level of recombinant Trx-MOG.

SDS-PAGE and western blotting

SDS-PAGE was used to examine the expression amount of Trx-MOG. After being resuspended in 100μL of 4XSDS sample buffer and 300 μL of phosphate-buffered saline (PBS), the heattreated samples (for 7 minutes at 90 to 100 °C) were loaded onto a 15.5% SDS-PAGE gel (stacking gel voltage: 80 V and separating gel voltage: 120 V). The gel was stained with Coomassie brilliant blue G-250 after electrophoresis. For protein quantification, ImageJ software (Version 1.41, NIH, USA) was employed to assess the SDS-PAGE gel' images.[15] For western blot analysis, proteins were electro-transferred (at a voltage of 90 V for 120 min) from gels to polyvinylidene difluoride (PVDF) membranes with the Mini Trans-Blot Cell (Bio-Rad, USA). The transplanted membrane was then blocked for 1 hour in tris-buffered saline-tween (TBST) containing 5% nonfat milk. After three TBST washes, the membrane was treated overnight with Mouse monoclonal anti-poly-HIS (1:10,000) (Sigma, UK). After another wash, the membrane was immersed in horseradish peroxidase-labeled goat anti-mouse IgG as the secondary antibody (1:5,000) (Sigma, UK) for 2 hours. Protein bands were detected by using 3,3′-diaminobenzidine solution (DAB; Sigma, UK).[13]

Quantification of Trx-MOG protein

Using bovine serum albumin (BSA, Atocell, Austria) as the standard, the bicinchoninic acid (BCA, Takara, Japan) test was utilized to evaluate the amounts of total proteins. BCA reagents A and B were combined in a 100:1 ratio to create the working solution prior to measurement. The methodology was followed in the preparation of BSA standard solution dilutions. Samples and BSA standard solution at each dilution were added to a microtiter plate in a volume of 25 μL. There were two measurements made for every concentration. The microtiter plate was filled with 200 μL of the working solution, and it was mixed immediately. The plate was incubated at 37 °C for 30 minutes. A microplate reader (Biotek Instruments, Power Wave XS, Germany) was used to detect the absorbance at 562 nm. Based on the concentrations of BSA standard samples, the standard curve was created. The standard curve was used as a guide to calculate the total protein concentration. After electrophoresis (12% SDS-PAGE), ImageJ software was used to assess the band intensities as well as the percentage of Trx-MOG protein as a proportion of total protein. The concentration of Trx-MOG protein was calculated based on data obtained from BCA test and ImageJ software analysis.[15]

Results

Construction of the recombinant plasmid and protein expression

The gene encoding the N-terminal extracellular IgG V-like MOG domain was cloned into the *Nco*I and *Hind*III sites of the pET32a(+) vector. The pET32a(+)-MOG plasmid was effectively generated, according to PCR assay (Figure 2). The Trx-MOG protein was expressed in LB broth (291 aminoacids, ~ 32.5 kDa), as demonstrated by SDS-PAGE

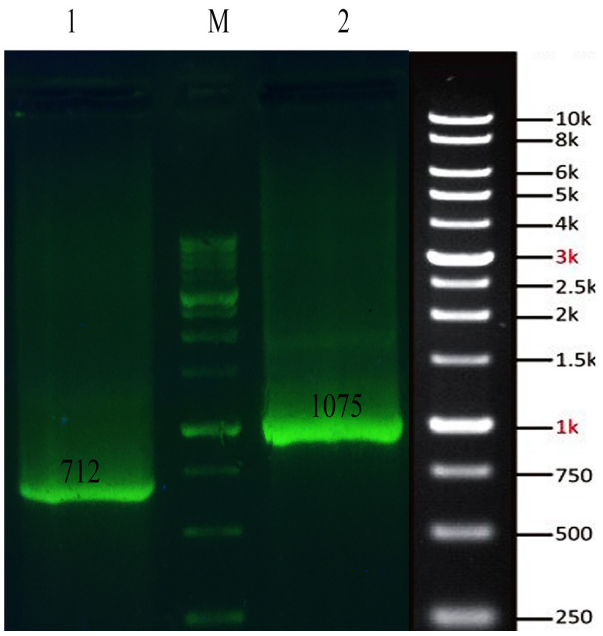

Figure 2. Analysis of the pET32a(+)-MOG plasmid by utilizing the polymerase chain reaction. The figure shows PCR products from control (pET32a(+), 712 bp, lane 1) and recombinant plasmid (pET32a(+)-MOG, 1075bp, lane 2) using universal primers (T7 promoter and T7 terminator primers), confirming the insertion of the target gene into the vector. Lane M: DNA marker (1 kb).

(Figure 3A). The recombinant protein was validated in western blotting using monoclonal antibodies (Figure 3B).

Optimization of the Trx-MOG expression using RSM

The RSM-BBD approach was effectively used to study the impacts of post-induction temperature, IPTG concentration, pre-induction optical density, and post-induction duration on Trx-MOG synthesis in LB broth medium. Table 2 shows the results of a three-level BBD with 29 runs (Figure S1 in Supplementary Data). Based on the obtained results, we found that the experimental data were described best with quadratic equation as follows:

$$Y=[\text{Trx-MOG }(\mu g)]= 682.70 - 239.22\ A - 9.08\ B + 195.53\ C - 57.86\ D - 270.93\ AC + 160.08\ BC - 130.15\ BD + 163.30\ A^2 + 281.60\ B^2 - 121.97\ C^2 + 31.59\ D^2$$

$$(R^2 = 0.8638,\ R^2_{adj} = 0.7756)$$

Where Y denotes the Trx-MOG (µg) protein content and A, B, C, and D denote the temperature, pre-induction optical density, time, and inducer concentration, respectively. Based on the results shown in Table 2, a broad range of findings (395.21 to 1600.09 µg) for the recombinant protein amount was achieved for all 29 trials. The highest protein content (1600.09 µg) was achieved when cells were incubated for 24 hours at 23 °C with 0.7 mM IPTG.

Statistical analysis

Utilizing the Design-Expert program, analysis of variance (ANOVA) verified the quadratic regression model for optimizing Trx-MOG production. Table 3 displays the findings of ANOVA values. The quadratic model's F-value was significant, and ANOVA revealed that there was only a 0.01% probability that the model's F value could have resulted from noise (9.8). Moreover, the P value (0.4765) of the selected model showed insignificant lack of fit value. The r^2 value of 0.8638 for the quadratic regression model demonstrated the model's applicability and showed a strong correlation between the actual and predicted results. In addition, an acceptable level of correlation between the predicted and actual findings was shown by a good agreement (0.2) between the adjusted $r^2 = 0.7756$ and predicted $r^2 = 0.5400$. The pattern depicted in Figure 4A further validated this association. The studentized residuals' normal probability plot was another useful tool for assessing the model's accuracy and predictability. As

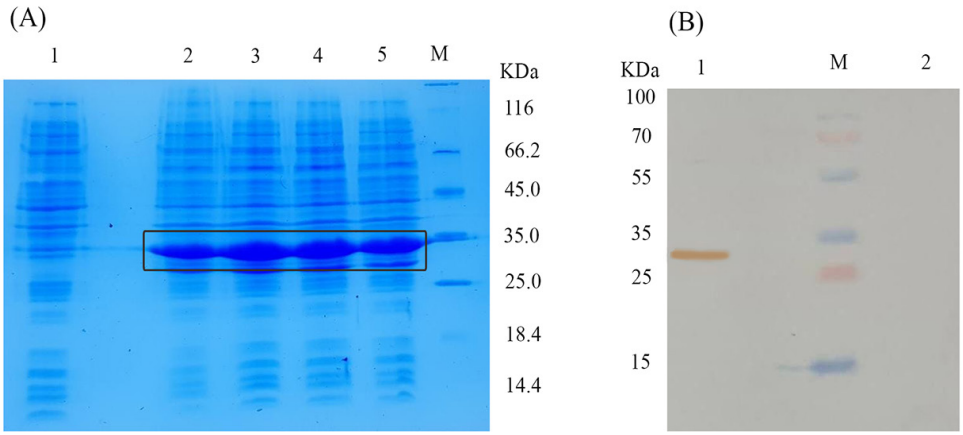

Figure 3. Expression and western blotting analyses of the expressed Trx-MOG protein. (A) According to SDS-PAGE, protein of the expected size (~ 32.5 kDa) was detected. Lane M, protein marker (14.4-116 kDa); Lane 1, total protein from E. coli strain containing pET32a(+)-MOG plasmid before induction; Lanes 2-5, total cell lysate of Trx-MOG -expressing strain induced in OD600 = 0.6-0.8 with 1 mM IPTG at 37 °C for 2, 4, 6 and 24 hours in LB broth medium; (B) Using a 6x His-tag, the expressed protein (~32.5 kDa) was validated in western blotting with an anti-His-tag monoclonal antibody. Lane M, protein ladder (10-250 kDa); lane 1, induced Trx-MOG protein in LB, lane 2, uninduced bacterial lysate.

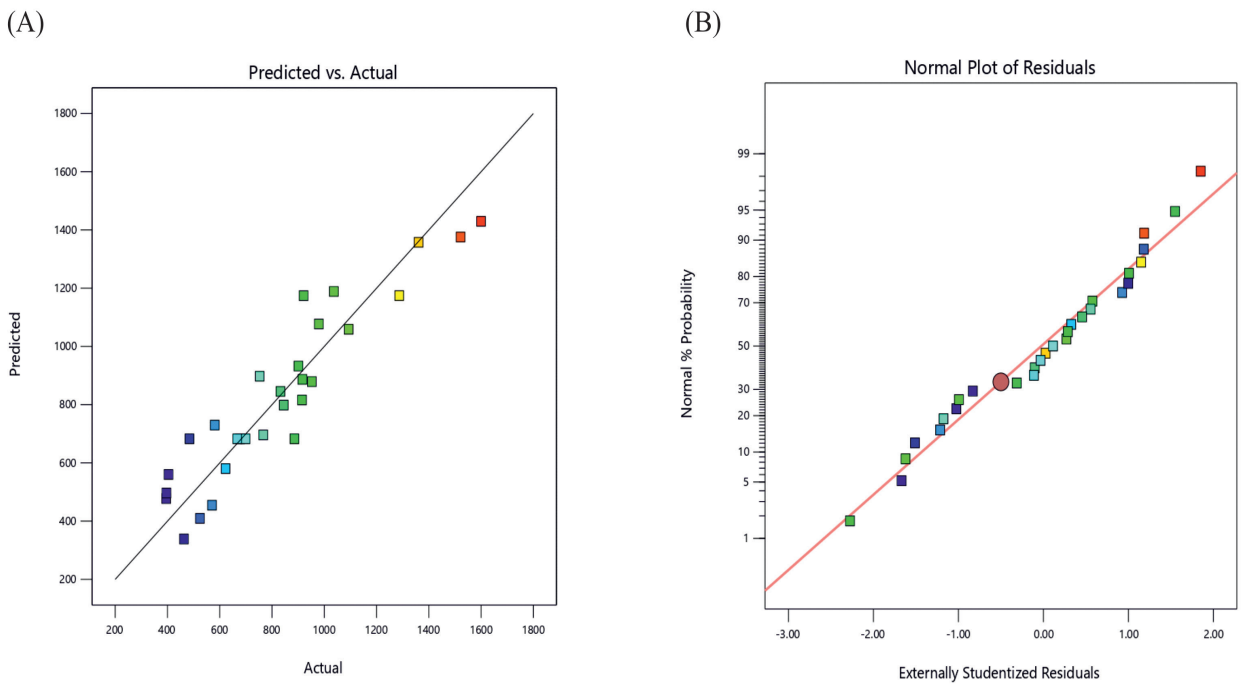

Figure 4. Regression model adequacy diagnostic plots. (A) Diagnostic plot of predicted versus actual responses for Trx-MOG expression; (B) Normal probability plot of internally studentized residuals for Trx-MOG production.

shown in Figure 4B, the significance and adequacy of the model were confirmed by the data points' linear trend and normal distribution. Thus, ANOVA findings showed that the quadratic model developed in this work suited the experimental data quite well and, as a consequence, could be utilized to explore the design space.

Factors influencing the recombinant production of Trx-MOG

A and C are significant model terms ($P < 0.05$), based on the results of the ANOVA (Table 3). Due to its large sum of squares (6.87×10^5), low P-value (<0.0001), and high F value (29.56), the post-induction temperature has the most impact, as Table 3 illustrates.

Table 3. ANOVA of the experimental results.

Source	Sum of Squares	df	Mean Square	F-value	*P*-value Prob > F*
Model	2.50×10^6	11	2.28×10^5	9.8	< 0.0001
A-Temp	6.87×10^5	1	6.87×10^5	29.56	< 0.0001
B-OD	990.41	1	990.41	0.0426	0.8389
C-Time	4.59×10^5	1	4.59×10^5	19.75	0.0004
D-IPTG	40166.64	1	40166.64	1.73	0.206
AC	2.94×10^5	1	2.94×10^5	12.64	0.0024
BC	1.03×10^5	1	1.03×10^5	4.41	0.0509
BD	67758.17	1	67758.17	2.92	0.1059
A^2	1.73×10^5	1	1.73×10^5	7.45	0.0143
B^2	5.14×10^5	1	5.14×10^5	22.14	0.0002
C^2	96502.74	1	96502.74	4.15	0.0574
D^2	6474.97	1	6474.97	0.2787	0.6044
Residual	3.95×10^5	17	23231.01		
Lack of Fit	3.14×10^5	13	24134.23	1.19	0.4765
Pure Error	81182.18	4	20295.55		
Cor Total	2.90×10^6	28			

A, temperature (°C); B, optical density 600 (nm); C, Time (h); D, isopropyl-b-D-thiogalactopyranoside (IPTG, mM); *, value of Prob > F less than 0.0001 indicates that the model terms are significant. In this model A, C, AC, BC, A^2, B^2, and C^2 are significant model terms.

Following that, the post-induction time (4.59×10^5, 19.75, and 0.0004, respectively) is the second important parameter. Inducer concentration and pre-induction optical density had no impact on Trx-MOG production when compared to other parameters. The square terms of pre-induction optical density, post-induction time, and temperature were also significant (P = 0.0002, P = 0.0574, and P = 0.0143, respectively). 3D surface plots show the interaction effects of parameters on Trx-MOG expression level (Figure 5A-C). The influence of the two most important factors, temperature and time, on the production of Trx-MOG is displayed in Figure 5A. The interaction (P = 0.0024) between these two terms was significant, according to the ANOVA results. Figure 5A demonstrates that increasing time has a greater impact on the response at low temperatures, but it has a lesser effect at higher temperatures. Moreover, at high post-induction time, increasing temperature significantly decreases the protein expression level. The interaction between pre-induction OD and time is significant based on the low P-value (0.0509). Figure 5B indicates that increasing the post-induction time enhances Trx-MOG protein production, notably at higher OD_{600}s. Furthermore, the production of recombinant protein decreases with increasing cell density until approximately OD_{600} equals 0.7, after which it increases. Figure 5C depicts the effect of IPTG concentration and cell density before induction on Trx-MOG synthesis. According to Figure 5C, increasing IPTG concentration at high cell density leads to decrease in Trx-MOG expression level. The model also predicted that the optimal culture conditions for maximum Trx-MOG production would be inducer concentration of 0.491 mM, time of 23.878 h, OD_{600} of 0.8, and temperature of 23 °C. Overall, the model was validated by expressing Trx-MOG at the optimal predicted conditions (three times). As a consequence, the recombinant Trx-MOG protein yielded 1639.593 µg, which was close to the amount (2000.419 µg; Figure 6) predicted by the model. As a result, the BBD model's accuracy and suitability for recombinant Trx-MOG synthesis were validated.

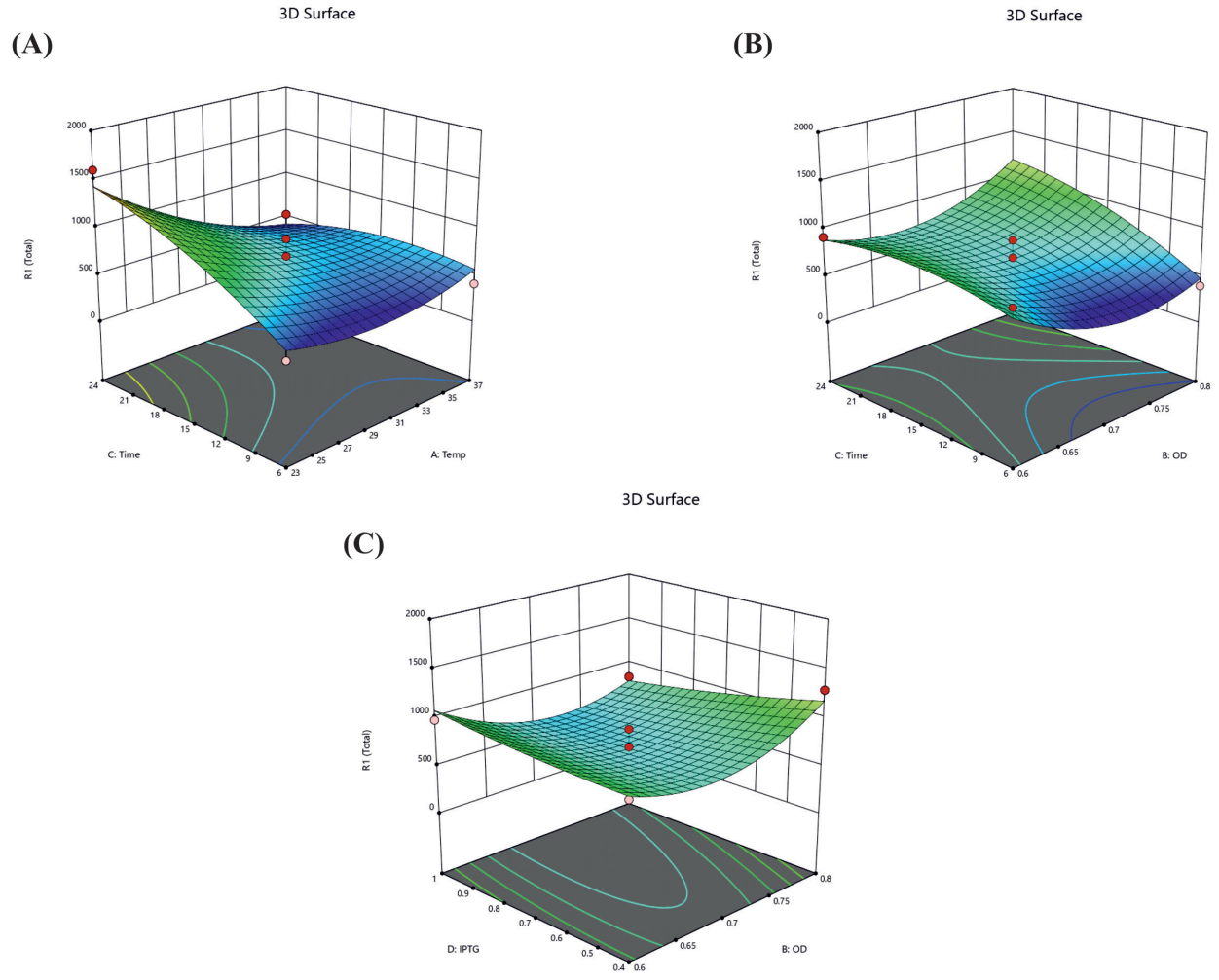

Figure 5. The 3D response surface plots of Trx-MOG expression level considering (A) Post-induction time and temperature; (B Post-induction time and cell density (OD600), (C) IPTG concentration and cell density (OD600), on Trx-MOG expression level.

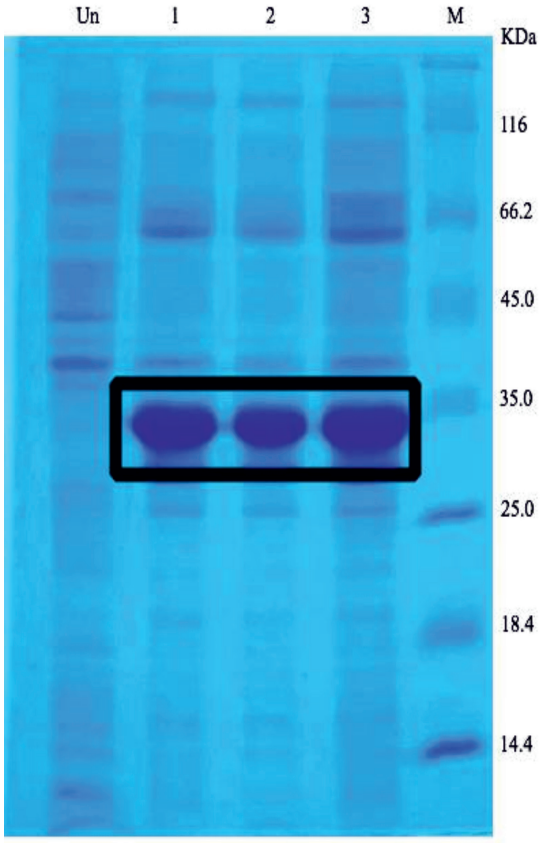

Figure 6. SDS-PAGE analysis to characterize the optimum conditions. Lane M, protein marker (14.4-116 kDa); lane Un, total protein from E. coli strain containing pET32a(+)-MOG plasmid before induction; lanes 1-3, three repeats of the experiments carried out in LB medium under the optimum conditions. Trx-MOG protein of the expected size (~ 32.5 kDa) was detected.

Discussion

A complicated multidimensional illness, MS, is characterized by neurodegeneration and autoimmune inflammation in the central nervous system (CNS). Animal models are critical for both MS research and the development of treatment methods. Experimental autoimmune encephalomyelitis (EAE), the most widely used MS model, can be produced by myelin oligodendrocyte protein (MOG).[16] The first attempts to extract native MOG from either murine or porcine brain tissues, as described by Ohashi *et al.*,[17] failed to provide enough protein for encephalitogenic experiments.The use of *E. coli* as a model organism for recombinant protein production provides a straightforward method for high-level heterologous protein manufacturing.[5] Here, employing the RSM-BBD approach, the expression level of the Trx-MOG fragment was optimized. Using this method, a suitable F-value and a low P-value ($F= 9.8$, $P < 0.0001$) confirm that the developed model are significant. Its insignificant lack of fit values further supported the chosen model's adequate prediction performance. According to previous studies, the Box-Behnken design is more effective than both full-factorial and the central composite designs.[6] As an example, the fragmented antibody named 4D5MOC-B was optimally produced in the SHuffle™ T7 strain by using the BBD approach.[13] Moreover, utilizing the RSM-BBD technology, culture conditions for anti-keratin ScFv TS1-218 production was optimized with a 21-fold increase by Jafari and coworkers.[11] They assessed how pH, temperature, and methanol concentration affected protein expression. Additionally, using BBD-RSM approach, Emamipour *et al.*[12] successfully optimized expression and purification of DsbA-IGF1.

Our results revealed that the time and temperature could significantly influence the Trx-MOG expression level. Additionally, at low temperatures, increasing the post-induction time greatly increases the amount of recombinant protein. While 37 °C is frequently employed temperature in protein expression due to faster growth of *E. coli* and higher efficiency in transformation, our study shows the potential advantages of lower temperatures combined with long post-induction times for producing higher protein contents. Lower temperatures give proteins more time to fold correctly, which reduces aggregation and promotes the expression of more complicated or larger proteins. Additionally, lower temperatures enable bacterial chaperones to aid in the folding procedure, increasing the amount of correctly folded proteins produced. Lower temperatures can also help to prevent protein degradation, as higher temperatures can activate endogenous proteases that can degrade proteins and reduce yields.[18,19] Moreover, it was revealed that a long incubation time serves as crucial for optimum Trx-MOG expression in *E. coli*. However, short post-induction times (less than 8 h) were suitable for some recombinant proteins.[20] The type of protein and the host therefore influence the ideal post-induction period.
Optimal protein production in *E. coli* strongly depends on the optical density of the bacterium prior to IPTG induction. In general, the optimal OD value for induction lies between 0.6 and 0.8, albeit it may change based on the particular protein and its properties. To ensure sufficient biomass and cellular activity and to prevent problems like protein aggregation or misfolding within the cells, it is essential to optimize the OD value. Higher OD values have the potential to cause metabolic stress and to cause cellular reactions such as the proteases synthesis. So, lower OD values are suitable for proteins that are unstable, prone to precipitate, or vulnerable to cleavage.[5] According to our findings, when the cell density prior to induction is 0.8, which is close to the late-log phase, the protein can be expressed at its optimum level. The majority of recombinant bacteria grow rapidly in this phase, which is ideal for the expression of recombinant proteins. Consistently, according to Galloway and colleagues, the expression level of ACF64 significantly increased when protein production was induced at the late-log phase.[21]

The optimum inducer concentration must be found because low inducer concentrations may not allow for protein expression, and excessive concentrations may be harmful to bacteria.[22] According to statistical analysis

and 3-D plots, the optimal IPTG concentration for high yield Trx-MOG synthesis was 0.491 mM. Furthermore, the expression of Trx-MOG steadily decreased as IPTG concentrations rose above 0.5 mM, particularly at higher optical densities, indicating IPTG's toxicity to *E. coli*. Consistent with our findings, by raising the IPTG concentration over 0.6 mM, it was shown that the production of recombinant synthase significantly decreased.[23] Moreover, *E. coli* expressed more recombinant Man i 1 (rMan i 1), a mango allergen, at the lower IPTG concentration (0.1 mM).[24] The IPTG adverse impact may be attributed to the increased metabolic burden and bacterial protease activation, which may destroy heterologous proteins. In response to the activation of protein synthesis in the presence of excessive IPTG, an increase in rRNA synthesis has been observed in cells. The increased host cell metabolite load results from this change in RNA production, which in turn redirects energy sources. Moreover, metabolite load occurs when the recombinant protein is expressed and stabilized using host cell ATP or GTP as well as specific amino acids.[25] The type of expression host also influences the ideal IPTG concentration. For example, optimum concentration of IPTG for achieving high amount of antiEpEX-scFv fragment was 0.8 mM and 0.5 mM in *E. coli* BW25113 (DE3) and BL21 (DE3) strains respectively.[22] Ahmadzadeh *et al.*[15] reported similar results. They showed that although different concentrations of IPTG did not significantly affect the expression level of anti-HER2 scFv in SHuffle™ T7, a low inducing concentration (0.25 mM) resulted in the production of high amounts of that protein in *E. coli* BL21 (DE3).

Conclusion

In summary, this study provides important new information about the RSM-optimized N-terminal extracellular MOG synthesis in *E. coli*. A total of 29 trials were planned to examine the interactions of four key influencing factors including post-induction time, inducer concentration, pre-induction optical density, and post-induction temperature on the Trx-MOG expression level in *E. coli* BL21 (DE3) using the RSM-BBD approach. Finally, Design Expert software suggested a quadratic model based on the testing data. The two variables A (post-induction time) and C (post-induction temperature) were shown to have a significant impact on protein synthesis using the suggested model. The following optimum growth conditions for high yield Trx-MOG synthesis could be achieved: cell density prior to induction (OD_{600}) of 0.8, inducer concentration of 0.491 mM, and 23 °C for 23.878 hours. The optimum protein concentration was attained (163.96 µg/mL) and was within the range of (200.04 µg/mL), which was the value predicted.

Author Contributions

Maryam Radmard: Investigation, Methodology, Visualization. Atieh Hashemi: Conceptualization, Visualization, Supervision, Resources, Writing - Original Draft.

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Supplementary Data

Supplementary data (Figure S1) are available at https://doi.org/10.34172/PS.2024.1.

References

1. Glatigny S, Bettelli E. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Cold Spring Harb Perspect Med. 2018;8(11):1079-106. doi:10.1101/cshperspect.a028977
2. Bäckström BT. A rapid, simple, and standardized homogenization method to prepare antigen/adjuvant emulsions for inducing experimental autoimmune encephalomyelitis. J Vis Exp. 2022;2022(190):e64634. doi:10.3791/64634
3. Miller SD, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol. 2010;88(1):15. doi:10.1002/0471142735.im1501s77
4. Peng Z, Zhang L, Wang H, He X, Peng X, Zhang Q, et al. Experimental autoimmune encephalomyelitis (eae) model of cynomolgus macaques induced by recombinant human mog1-125 (rhmog1-125) protein and mog34-56 peptide. Protein Pept Lett. 2017;24(12):1166-78. doi:10.2174/0929866524666171110093626
5. Francis DM, Page R. Strategies to optimize protein expression in *E. coli*. Curr Protoc Protein Sci. 2010;61:5.24.1-5.9. doi:10.1002/0471140864.ps0524s61
6. Jurado P, de Lorenzo V, Fernández LA. Thioredoxin fusions increase folding of single chain fv antibodies in the cytoplasm of escherichia coli: Evidence that chaperone activity is the prime effect of thioredoxin. J Mol Biol. 2006;357(1):49-61. doi:10.1016/j.jmb.2005.12.058
7. Staib L, Birebent B, Somasundaram R, Purev E, Braumüller H, Leeser C, et al. Immunogenicity of recombinant ga733-2e antigen (co17-1a, egp, ks1-4, ksa, ep-cam) in gastro-intestinal carcinoma patients. Int J Cancer. 2001;92(1):79-87. doi:10.1002/1097-0215(200102)9999:9999<::AID-IJC1164>3.0.CO;2-J
8. Salehinia J, Sadeghi HMM, Abedi D, Akbari V. Improvement of solubility and refolding of an anti-human epidermal growth factor receptor 2 single-chain antibody fragment inclusion bodies. Res Pharm Sci. 2018;13(6):566. doi:10.4103/1735-5362.245968
9. Malekian R, Jahanian-Najafabadi A, Moazen F, Ghavimi R, Mohammadi E, Akbari V. High-yield production of granulocyte-macrophage colony-

stimulating factor in e. Coli bl21 (de3) by an auto-induction strategy. Iran J Pharm Res. 2019;18(1):469-78. doi:10.1016/j.pep.2019.04.002
10. Malekian R, Sima S, Jahanian-Najafabadi A, Moazen F, Akbari V. Improvement of soluble expression of gm-csf in the cytoplasm of escherichia coli using chemical and molecular chaperones. Protein Expr Purif. 2019;160:66-72. doi:10.1016/j.pep.2019.04.002
11. Jafari R, Sundström BE, Holm P. Optimization of production of the anti-keratin 8 single-chain fv ts1-218 in pichia pastoris using design of experiments. Microb Cell Factories. 2011;10(1):1-8. doi:10.1186/1475-2859-10-34
12. Emamipour N, Vossoughi M, Mahboudi F, Golkar M, Fard-Esfahani P. Soluble expression of igf1 fused to dsba in shuffle™ t7 strain: Optimization of expression and purification by box-behnken design. Appl Microbiol Biotechnol. 2019;103:3393-406. doi:10.1007/s00253-019-09719-w
13. Behravan A, Hashemi A. Rsm-based model to predict optimum fermentation conditions for soluble expression of the antibody fragment derived from 4d5moc-b humanized mab in shuffle™ t7 *E. coli*. Iran J Pharm Res. 2021;20(1):254. doi:10.22037/ijpr.2020.114377.14822
14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. New York: Cold spring harbor laboratory press; 1989.
15. Ahmadzadeh M, Farshdari F, Nematollahi L, Behdani M, Mohit E. Anti-her2 scfv expression in escherichia coli shuffle® t7 express cells: Effects on solubility and biological activity. Mol Biotechnol. 2020;62:18-30. doi:10.1007/s12033-019-00221-2
16. Nissen JC, Tsirka SE. Preclinical model of multiple sclerosis: Methods in autoimmune demyelination. Methods in cell biology: Elsevier; 2022. p. 67-86. doi:10.1016/bs.mcb.2021.12.009
17. Ohashi T, Yukitake M, Slavin A, Krieger J, Hafler DA. In vitro evidence that immunuaffinity-purified mog contains immunogenic quantities of contaminating mouse igg; techniques for producing ig-free mog. J Neuroimmunol. 2001;118(2):194-202. doi:10.1016/s0165-5728(01)00321-6
18. Vasina JA, Baneyx F. Recombinant protein expression at low temperatures under the transcriptional control of the major escherichia coli cold shock promoter cspa. Appl Environ Microbiol. 1996;62(4):1444-7. doi:10.1128/aem.62.4.1444-1447.1996
19. Miyake R, Kawamoto J, Wei Y-L, Kitagawa M, Kato I, Kurihara T, et al. Construction of a low-temperature protein expression system using a cold-adapted bacterium, *Shewanella* sp. Strain ac10, as the host. Appl Environ Microbiol. 2007;73(15):4849-56. doi:10.1128/AEM.00824-07
20. Vasina JA, Baneyx F. Expression of aggregation-prone recombinant proteins at low temperatures: A comparative study of theescherichia coli cspaandtacpromoter systems. Protein Expr Purif. 1997;9(2):211-8. doi:10.1006/prep.1996.0678
21. Papaneophytou CP, Rinotas V, Douni E, Kontopidis G. A statistical approach for optimization of rankl overexpression in *Escherichia coli*: Purification and characterization of the protein. Protein Expr Purif. 2013;90(1):9-19. doi:10.1016/j.pep.2013.04.005
22. Soulari RN, Basafa M, Rajabibazl M, Hashemi A. Effective strategies to overcome the insolubility of recombinant scfv antibody against epcam extracellular domain in *E. coli*. Int J Pept Res Ther. 2020;26(4):2465-74. doi:10.1007/s10989-020-10044-4
23. Zheng P, Sun X, Guo L, Shen J. Cloning, expression, and characterization of an acetolactate synthase (ALS) gene from *Anabaena azotica*. Process Biochem. 2015;50(9):1349-56. doi:10.1016/j.procbio.2015.05.027
24. Tsai W-C, Wu T-C, Chiang B-L, Wen H-W. Cloning, expression, and purification of recombinant major mango allergen man i 1 in *Escherichia coli*. Protein Expr Purif. 2017;130:35-43. doi:10.1016/j.pep.2016.06.009
25. Lim K-P, Li H-B, Nathan S. Expression and purification of a recombinant scfv towards the exotoxin of the pathogen, burkholderia pseudomallei. J Microbiol. 2004;42(2):126-32.