Original Article

Statistical optimization of culture conditions for expression of recombinant humanized anti-EpCAM single-chain antibody using response surface methodology

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Abstract

Background and purpose: The epithelial cell adhesion molecule (EpCAM), is one of the first cancer-associated markers discovered. Its overexpression in cancer stem cells, epithelial tumors, and circulating tumor cells makes this molecule interesting for targeted cancer therapy. So, in recent years scFv fragments have been developed for EpCAM targeting.
Experimental approach: In this study, an scFv against EpCAM extracellular domain (EpEX) derived from 4D5MOC-B humanized mAb was expressed in *Escherichia coli* k12 strain, and in order to obtain the optimum culture conditions in chemically defined minimal medium, response surface methodology (RSM) was employed. According to the RSM-CCD method, a total of 30 experiments were designed to investigate the effects of various parameters including isopropyl-b-D-thiogalactopyranoside (IPTG) concentration, cell density before induction, post-induction time, and post-induction temperature on anti EpEX-scFv expression level.
Findings/Results: At the optimum conditions (induction at cell density 0.8 with 0.8 mM IPTG for 24 h at 37 °C), the recombinant anti EpEX-scFv was produced at a titer of 197.33 μg/mL that was significantly consistent with the prediction of the model.
Conclusion and implication: The optimized-culture conditions obtained here for efficient production of anti EpEX-scFv in shake flask cultivation on a chemically defined minimal medium could be applied to large-scale fermentation for the anti EpEX-scFv production.

Keywords: Anti EpEX; 4D5MOC-B humanized mAb; Response surface methodology; scFv.

INTRODUCTION

The epithelial cell adhesion molecule (EpCAM) or CD326 was one of the first discovered-cancer-associated biomarkers. Observations show that EpCAM is highly expressed on various types of solid tumors, including carcinomas of the breast, ovarian, lung, colon, and pancreatic cancer and also in squamous cell carcinoma of the head and neck (1). Based on the role of EpCAM as a signaling receptor, and its overexpression ability on tumor cells, it can be used as an attractive target molecule for immunotherapy as well as a diagnostic cancer marker (2). In recent years antibody fragments, particularly small recombinant single-chain variable fragments (scFvs) have been widely used for EpCAM targeting (3). ScFv is a small antibody fragment composed of variable regions of light and heavy chains of an antibody connected by a flexible peptide linker (4). These molecules have advantages over their parental antibodies including better tumor penetration, faster blood clearance, lower retention times in nontarget tissues, and reduced immunogenicity (5).

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Until now, different scFvs have been generated as anti-EpCAM agents but failed due to their poor *in vivo* stability. *in vivo* studies showed high thermal stability, good folding, and excellent tumor targeting for an anti EpEX-scFv derived from 4D5MOC-B humanized mAb (3). Due to stability features, this scFv can be highly applicable in the targeted delivery of drugs, toxins, and radioisotopes as well as diagnostic purposes (6). *Escherichia coli* has always been the preferred microbial cell factory for the production of recombinant proteins (7,8). Moreover, it is a suitable host for the high-level production of non-glycosylated antibody fragments like fragment scFvs (9,10). For large-scale fermentations, using chemically defined-minimal media makes one capable to determine requirements for growth and product formation, which will be possible by controlled feeding strategies *via* systematically adding or omitting chemical species from the formulation. In addition, interactions between undefined components of a complex media will be minimized and culture conditions will be reproducible (11). On the one hand, removing unknown compounds during downstream processes is not required when using defined-minimal media, and besides, defined-minimal media is less expensive than complex ones, therefore defined media will be more interesting for large-scale fermentations (12). However, low yields of the expressed recombinant proteins in minimal media have been a bottleneck for industrial applications.

Response surface methodology (RSM), which is based on the full factorial central composite design (CCD) has been used to optimize the production of various recombinant proteins in recent years (13). RSM is a statistical method in which specific factors are selected to achieve the favorable response that will be effective by simultaneously changing several variables based on a minimum number of experiments (14).

To circumvent the low-production yields in defined-minimal media, this study applied RSM-CCD methodology to find the optimum culture conditions to overproduction of the anti EpEX-scFv derived from 4D5MOC-B humanized mAb in *Escherichia coli* (*E. coli)* BW25113(DE3) considering parameters such

as induction temperature, cell density of induction time, post-induction time, and inducer concentration. Although a very stable 4D5MOC-B scFv fragment was previously expressed in *E. coli* B strains (15), the expression of this scFv in the cytoplasmic environment of *E. coli* BW25113(DE3), an *E. coli* K12 derived strain, has not been studied yet. While due to higher yields of expressed proteins, *E. coli* B strains are more commonly preferred as hosts compared to *E. coli* K12 derived strains, some obstacles such as plasmid loss can limit their utilization for large-scale production purposes (16). So, in the present study, we provided the first report, to our knowledge, for optimizing culture conditions for efficient production of 4D5MOC-B scFv fragment in *E. coli* BW25113(DE3) on a chemically defined-minimal medium, which could be applied to large-scale fermentation for the anti EpEX-scFv production.

MATERIALS AND METHODS

Bacterial strains and plasmid

E. coli strain (DH5α) was used as a cloning host for plasmid preparation. The Keio's collection parental strain BW25113 (rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 λ (DE3)), gifted from Prof. Dr. Silke Leimkühler, University of Potsdam, Potsdam, Germany, (17) was used as the bacterial host for the expression of the recombinant scFv. The pETDuet-1 co-expression vector (gifted from Dr. Bandehpour, Shahid Beheshti University of Medical Sciences, Tehran, Iran) was used as a vector for cloning the scFv gene. All restriction enzymes were provided by Thermo Fisher Scientific (USA). DNA fragments were purified from agarose gel using the gel extraction kit (Roche Diagnostics GmbH, Germany).

Chemicals and growth media

M9 minimal medium (1x) including 3.37 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.8 mM NaCl, 0.93 mM NH_4Cl, 0.1 mL 1000x trace metals mixture (Teknova, USA), 0.1 mM $CaCl_2$ (Merck, Germany), 2 mM $MgSO_4$ (Merck, Germany), was supplemented with

4 g/l D-glucose (Sigma-Aldrich, Germany), 0.5 mM thiamine hydrochloride (Sigma-Aldrich, Germany) and ampicillin (100 μg/mL). The composition of the Luria-Bertani (LB) medium was 1% (w/v) tryptone (Merck, Germany), 0.5% (w/v) yeast extract (Merck, Germany), and 1% (w/v) NaCl (Merck, Germany). All other chemicals were of analytical grade and obtained from Merck.

Construction of pETDuet-anti EpEX-scFv

The pGH vector harboring the synthesized codon-optimized anti EpEX-scFv gene fused with a C-terminal hexahistidine tag (provided by Generay Biotech Co, China) was digested with *XhoI* and *NdeI*. The purified gel extracted anti EpEX-scFv fragment was inserted into the *XhoI/NdeI* restriction sites located within multiple cloning site 2 (MCS2) of pETDuet-1 vector to construct the recombinant expression vector pETDuet-anti EpEX-scFv (Fig. 1). Chemically competent *E. coli* (DH5α) cells were transformed with pETDuet-anti EpEX-scFv plasmid using the heat shock method. theThe transformed cells were grown at 37 °C overnight on LB plates containing 100 μg/mL of ampicillin. Plasmids were isolated from positive clones using a plasmid extraction kit (Vivantis, Malaysia). The restriction digestion assay and sequencing on both strands were used to confirm the correct frame of the cloned fragment (Macrogen, Korea).

Protein expression

For initial determination of the protein expression, using the heat shock method, the plasmid pETDuet-anti EpEX-scFv was transformed into competent *E. coli* BW25113 (DE3) cells and a single colony of *E. coli* BW25113 (DE3) harboring pETDuet-anti EpEX-scFv was cultured overnight at 37 °C in LB broth supplemented with 100 μg/mL ampicillin. Fifty mL of fresh LB medium was inoculated with 10% (v/v) of the overnight culture and then incubated at 37 °C until the optical density at 600 nm (OD600) reached 0.6. Then isopropyl-b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mmol/L. After 24 h of induction, cells were harvested by centrifugation (10000 *g*; 10 min at 4°C) and kept at -20 °C for subsequent analysis. In order to perform the optimization experiments in M9 minimal medium, *E. coli* cells were firstly pre-cultured in liquid LB medium until OD600 of the culture reached a range of 1.5 to 2. The cells were then gently spun down at 700 *g* for 15 min at 4 °C. The cell pellet was resuspended in 50 mL of the M9 minimal medium containing the 100 μg/mL ampicillin to obtain an initial OD600 between 0.07 and 0.1. This culture was used for each experiment that was implemented under different conditions of cultivation, as described in the experimental design section (18).
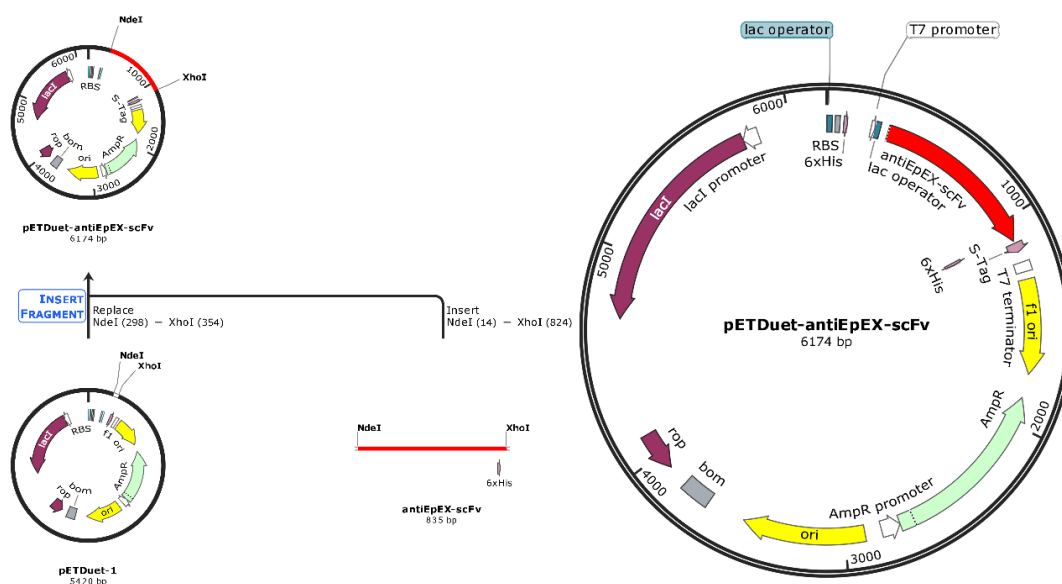

Fig. 1. Map of the pETDuet-anti EpEX-scFv expression vector: the gene encoding anti EpEX-scFv (~835 bp) was cloned into *Nde*I and *Xho*I sites downstream of the T7 promoter region of pETDuet-1 expression vector. Maps were designed with SnapGene Version 4.0.3.

Sample preparation

Cells were harvested by centrifugation of fermentation broth at 10000 *g*; 10 min at 4 °C. The pellet was resuspended in 20 mL of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mg/mL lysozyme, 1% Triton™ X-100). Then after 40 min incubation on ice, the cells were disrupted by sonication for 20 min (10/10-s on/off) at 400 W and centrifuged at 14,000 *g* for 30 min at 4 °C. The pellet was resuspended in 50 mL wash buffer containing (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton™ X-100) and then incubated at room temperature for 1 h followed by shaking at 250 rpm (19). The suspension was centrifuged at 14,000 *g* at 4 °C for 30 min, then the obtained supernatant was labeled as (S) fraction and the pellet was labeled as (IB) fraction. The expression level of the recombinant anti EpEX-scFv was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and western blotting

The expression level of anti EpEX-scFv was analyzed by SDS-PAGE. After resuspending in 100 μL of 4× SDS sample buffer, the samples were heated for 5 min at 90 to 100 °C and 10 μL of each sample was loaded on 15.5% SDS-PAGE gel. After the electrophoresis, the gel was stained by Coomassie brilliant blue G-250. Quantification of the expressed protein was accomplished by image analysis of the SDS-PAGE gel using ImageJ software (NIH, MD). For western blot analysis, the proteins were electrotransferred from the gel into the polyvinylidene difluoride (PVDF) membrane using a wet Transblot (Bio-Rad, USA). The transferred membrane subsequently was blocked in 5% non-fat milk in tris-buffered saline-tween (TBST) for 1. The membrane was then washed three times by TBST and incubated overnight in His-tag antibody (Sigma, UK). Again, it was washed, then the membrane was incubated in the secondary antibody (anti-mouse horseradish peroxidase-conjugated immunoglobulin, Sigma, UK) for 2 h and then detected using a solution of 3,3′-diaminobenzidine (DAB; Sigma, UK).

Quantification of anti EpEX-scFv protein

Bovine serum albumin (BSA) as a standard was used to calculate the concentration of the anti EpEX-scFv protein using the linear regression equation. In this quantification, discrete values of 0, 0.01, 0.05, 0.125, 0.250, and 0.500 mg/mL were used for preparing the standard concentrations of BSA as a range for drawing the standard curve. High-quality pictures were captured from the SDS-PAGE gels, and analyzed by the ImageJ software (20), then the recombinant anti EpEX-scFv protein levels were calculated using a linear regression equation $y = ax + b$ based on the area of the band. In this calculation, the level of the protein was the independent variable (x) and the area of the band was the dependent variable (y). The protein concentrations were determined by a bicinchoninic acid protein assay kit (Takara, Japan).

Experimental design

Using the factorial RSM-CCD methodology, the effects of four independent variables including inducer concentration, post-induction temperature, post-induction time, and cell density at induction time on the expression level of the recombinant protein were systematically examined in this study (Table 1).

Table 1. Independent variables and values of levels used in central composite design.

Variables	Levels				
	-α	-1	0	+1	+α
Post-induction time (h)	0	8	16	24	32
Cell density (OD600, nm)	0.5	0.6	0.7	0.8	0.9
Post-induction temperature (°C)	16	23	30	37	44
IPTG concentration (mmol/L)	0.2	0.4	0.6	0.8	1

IPTG, Isopropyl-b-D-thiogalactopyranoside.

Table 2. The central composite design of four variables and five levels.

Run	Time (h)	OD600 (nm)	Temperature (°C)	IPTG (mmol/L)	Anti EpEX-scFv expression level (µg/mL)
1	0	0	-α	0	11.97
2	-1	1	-1	1	22.14
3	1	1	-1	1	13.15
4	-1	1	-1	-1	60.82
5	-1	-1	-1	-1	44.8
6	-1	-1	-1	1	22.08
7	1	1	-1	-1	33.82
8	1	-1	-1	1	20.8
9	1	-1	-1	-1	24.32
10	0	0	0	0	118.55
11	0	0	0	0	86.95
12	0	0	0	0	106.8
13	0	0	0	0	105.05
14	0	-α	0	0	84.19
15	0	0	0	0	83.82
16	0	0	0	0	119.85
17	0	+α	0	0	138.22
18	0	0	0	-α	117.56
19	0	0	0	+α	65.77
20	+α	0	0	0	17.64
21	-1	1	1	1	114.05
22	-1	-1	1	1	95.5
23	1	1	1	1	201.86
24	1	-1	1	1	121.91
25	1	1	1	-1	181.58
26	-1	-1	1	-1	88.83
27	-1	1	1	-1	114.51
28	1	-1	1	-1	126.36
29	-α	0	0	0	0
30	0	0	+α	0	178.88

IPTG, Isopropyl-b-D-thiogalactopyranoside.

A four-factor-five-level CCD consisted of 16 factorial points, 8 axial points, and 6 RSM center point replicates was leading to a set of 30 experiments as presented in Table 2. Furthermore, all the experiments were performed in 250 mL Erlenmeyer flasks with 50 mL of M9 minimal medium (Table 2). Design of the experiments and analysis of the results were accomplished using the software package Design Expert version 11® (Stat-Ease Inc., Minneapolis, USA). Moreover, to evaluate the validity of the model presented in the results, the combination of tested variables leading to the maximum predicted level of the expressed anti EpEX-scFv was experimentally validated.

RESULTS

Construction of the recombinant plasmid and protein expression

The gene encoding anti EpEX-scFv (~835 bp) was cloned into *NdeI* and *XhoI* sites downstream of the T7 promoter region of pETDuet-1 expression vector (Fig. 1). Restriction digestion analysis and sequencing results demonstrated that the pETDuet-anti EpEX-scFv plasmid was constructed successfully (Fig. 2). In order to examine the expression level of the recombinant anti EpEX-scFv protein in *E. coli* strain BW25113 (DE3), the cell lysate prior to centrifugation, centrifugal supernatant and pellet fractions were analyzed by SDS-PAGE. These results demonstrated that the major part of the anti EpEX-scFv was expressed in aggregated forms (inclusion bodies) (Fig. 3A). According to bicinchoninic acid results and densitometric analysis of SDS-PAGE bands, a fairly good expression was detected up to 20% (450.430 µg/mL) of the total protein in *E. coli* strain BW25113 (DE3). Using a C-terminal histidine (6x His-tag), the expressed scFv was confirmed by anti-His-tag monoclonal antibody in western blotting (Fig. 3B).
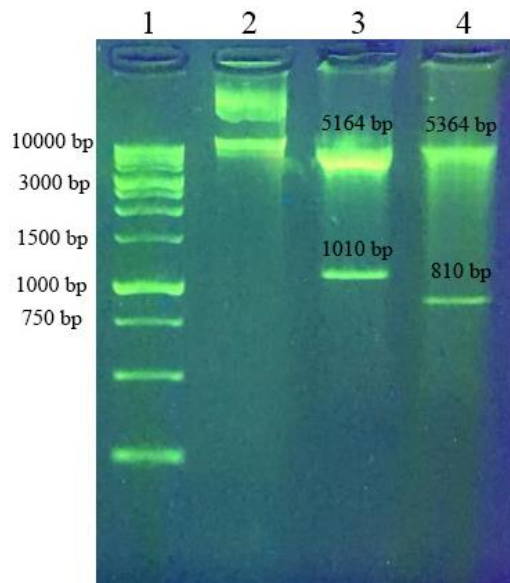
Fig. 2. Analysis of the recombinant plasmid pETDuet-anti EpEX-scFv by digestion reaction. Lane 1, DNA marker (1 kb); lane 2, plasmid pETDuet-1 (5420 bp); lane 3, recombinant plasmid pETDuet-anti EpEX-scFv digested with *NcoI*

The recombinant anti EpEX-scFv was also expressed in the defined-minimal medium. SDS-PAGE and western blotting analysis were shown that the target protein was successfully expressed in M9 minimal medium (Fig. 3A and B).

Optimization of the anti EpEX-scFv expression using RSM

RSM-CCD methodology was successfully utilized to investigate the individual and interaction effects of post-induction temperature, cell density before induction, IPTG concentration, and post-induction time on the anti EpEX-scFv production in M9 minimal medium. The five-level CCD with 30 runs was employed (Tables 1 and 2). The experimental levels of protein production were presented in Table 2. Based on the experimental data, the correlation between the response (expression level of anti EpEX-scFv (μg/mL)) and four different variables (post-induction temperature, cell density, IPTG concentration, and post-induction time) was described with a quadratic model represented by the following equation:

$$y = \left[antiEpEX - scFv \left(\frac{\mu g}{ml}\right)\right] = 103.501 + 8.21009\,A + 12.7899\,B + 47.0741\,C - 6.99159\,D + 4.75124\,AB + 17.3675\,AC + 3.00974\,AD + 10.1336\,BC - 0.927612\,BD + 6.64439\,CD - 23.0171\,A^2 + 2.5799\,AB^2 - 1.74094\,C^2 - 2.3044 D^2$$

($R^2 = 0.9617$, $R^2_{adj} = 0.9259$)
where, Y is the response (anti EpEX-scFv expression level), and A, B, C, and D are letters used for a post-induction time, cell density before induction, post-induction temperature, and IPTG concentration, respectively. Based on designed experiments, a wide range of results (0 to 201 μg/mL) was obtained for the expression level of the recombinant protein for all 30 experiments (Table 2). The highest total protein level (201.86 μg/mL) was obtained when cells were induced at 37 °C with 0.8 mM IPTG for 24 h.
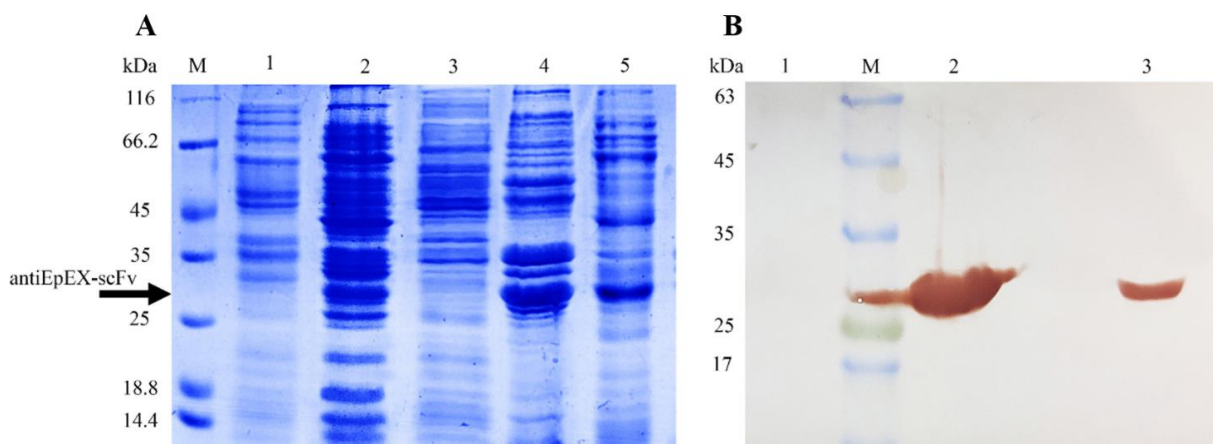

Fig. 3. (A) SDS-PAGE and (B) western blotting analyses of the expressed anti EpEX-scFv. (A) Lane M, unstained protein marker (14.4-116 kDa); 1, total lysate of *E. coli* BW25113 (DE3) harboring empty pETDuet-1; lanes 2-4, total lysate, supernatant, and inclusion body proteins extracted from *E. coli* BW25113 (DE3) expressing anti EpEX-scFv; lane 5, total lysate of *E. coli* BW25113 (DE3) expressing anti EpEX-scFv at the optimum condition in M9 medium. (B) Lane M, protein ladder (10-250 kDa); lane 1, total lysate of induced transformant *E. coli* BW25113(DE3) with empty vector; lane 2, induced anti EpEX-scFv protein in LB; lane 3, M9 media. *E. coli, Escherichia coli;* SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 3. ANOVA for the response surface quadratic model proposed for culture condition optimization for the anti EpEX-scFv production in M9 minimal medium.

Source	Sum of squares	df	Mean square	F-value	*P*-value Prob > F*
Model	83104.29	14	5936.02	26.90	< 0.0001
A	1617.73	1	1617.73	7.33	0.0162
B	3919.77	1	3919.77	17.76	0.0008
C	53183.24	1	53183.24	241.02	< 0.0001
D	1173.18	1	1173.18	5.32	0.0358
AB	361.19	1	361.19	1.64	0.2202
AC	4826.07	1	4826.07	21.87	0.0003
AD	144.94	1	144.94	0.6568	0.4304
BC	1643.05	1	1643.05	7.45	0.0155
BD	13.77	1	13.77	0.0624	0.8061
CD	706.37	1	706.37	3.20	0.0938
A²	14531.25	1	14531.25	65.85	< 0.0001
B²	182.57	1	182.57	0.8274	0.3774
C²	83.13	1	83.13	0.3767	0.5485
D²	145.66	1	145.66	0.6601	0.4292
Residual	3309.90	15	220.66		
Lack of Fit	2141.88	10	214.19	0.9169	0.5778
Pure Error	1168.01	5	233.60		
Correlation total	86414.19	29			

A, Time (h); B, optical density 600 (nm); C, temperature (°C); D, isopropyl-b-D-thiogalactopyranoside (IPTG, mM); *, value of Prob > F less than 0.0500 indicates that the model terms are significant. In this model A, B, C, D, AC, BC, and A² are significant model terms.
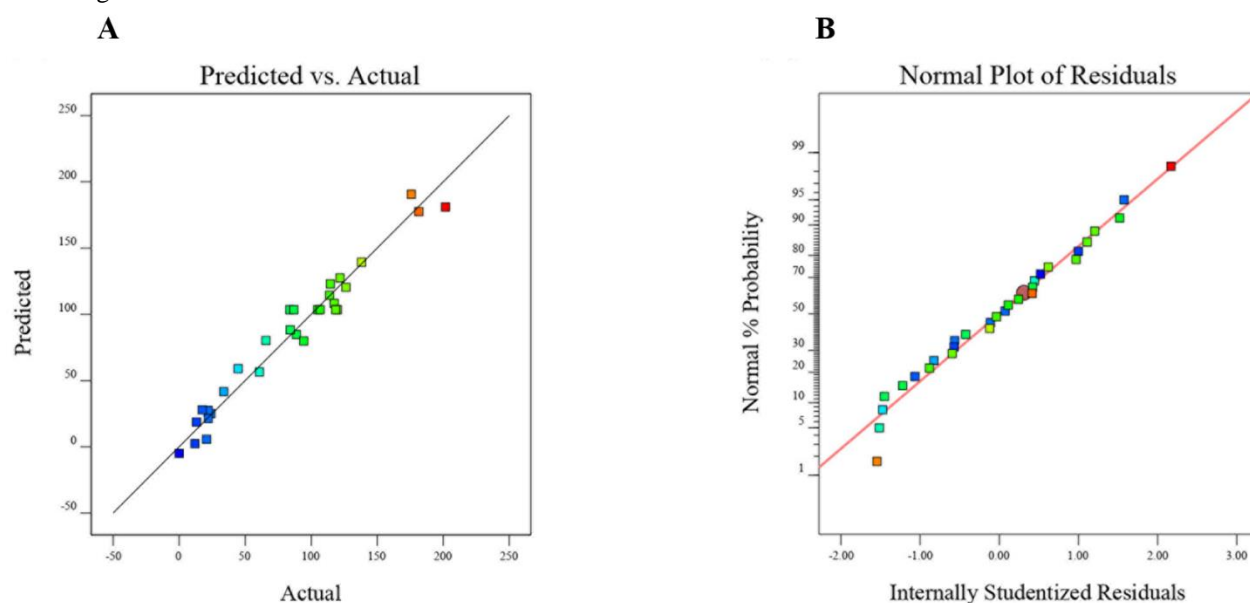

Fig. 4. Regression model adequacy diagnostic plots. (A) the plot of response surface methodology predicted values versus experimental responses for anti EpEX-scFv expression; (B) studentized residuals and normal (%) probability plot for anti EpEX-scFv production.

Statistical analysis of the model

The quadratic regression model for optimization of the anti EpEX-scFv production was confirmed by analysis of variance (ANOVA) using the Design-Expert software. The results of ANOVA values are represented in Table 3. ANOVA showed that the F-value of the quadratic model was high (26.90) and there was only a 0.01% chance that the F value of the proposed model could occur due to the noise. Moreover, the lack of fit F value of the selected model was not significant due to its corresponding *P* value (0.5778). The r^2 value for the quadratic regression model was found to be 0.9617, which showed a good correlation between the actual and the predicted values and confirmed the good suitability of the selected model. Moreover, a good agreement (< 0.2) between predicted r² = 0.8378 and the adjusted r^2 = 0.9259 confirmed a high degree of correlation between the predicted and the experimental values. This correlation was more

confirmed by the plot illustrated in Fig. 4A. The predictability and accuracy of the selected model were also effectively evaluated *via* the normal probability plot of the studentized residuals. As illustrated in Fig. 4B, the normal distribution and the linear trend of data points in the normal probability plot of the proposed quadratic model demonstrated its significance and adequacy. Thus, ANOVA results indicated that the quadratic model proposed in this study was fitted very well to the experimental data, and consequently could be used to navigate the design space.

Influence of parameters on the production of the recombinant anti EpEX-scFv

According to ANOVA results (Table 3), A, B, and C can be considered as significant model terms ($P < 0.05$). As represented in Table 3, the post-induction temperature is the most affecting parameter due to the high sum of square (53183.24), high F value (241.02), and low P-value (< 0.0001). Subsequently, the second significant parameter is cell density before induction (3919.77, 17.76, and 0.0008, respectively). Moreover, the third affecting parameter is post-induction time (1617.73, 7.33 and 0.0162, respectively). Compared to other parameters, IPTG concentration is the least affecting parameter on the anti EpEX-scFv production. The square terms of post-induction time are also significant ($P < 0.0001$). The interactive effects of independent variables on the expression level of anti EpEX-scFv are also studied using 3D surface plots (Fig. 5A-E). The dependency of IPTG concentration and post-induction temperature on the anti EpEX-scFv production is shown in Fig. 5A. No significant interaction between these two parameters is indicated by ANOVA ($P = 0.0938$; Table 3). According to Fig. 5A, temperature rise can lead to an increase in the production of anti EpEX-scFv but increasing IPTG concentration is not effective. Additionally, as depicted in Fig. 5B, the production of anti EpEX-scFv is improved by increasing the post-induction time while upon an increment in the IPTG concentration, there is no significant increase in protein production. The effect of the two most affecting parameters, namely post-induction time and post-induction temperature on the scFv production is depicted in Fig. 5C. ANOVA

results demonstrated a significant interaction ($P = 0.0003$) between these two parameters. From Fig. 5C, it is clear that protein expression increases significantly with both post-induction temperature and post-induction time. According to Table3, the term of interaction between post-induction temperature and cell density before induction is also significant ($P = 0.0155$). As shown in Fig. 5D, increasing the OD600 at low temperature has no effect on the anti EpEX-scFv production, whereas the maximum level of protein expression is obtained with the highest level of both temperature and OD600, simultaneously. The high P-value of the coefficient post-induction time and cell density before induction of 0.2202 indicated that the interaction between an incubation time and cell density before induction is not significant (Table 3). Figure 5E declares that although increasing the value of OD600 has a low effect on the anti EpEX-scFv production at lower incubation times, it will be more effective at higher incubation times. Finally, the dependency of IPTG concentration and cell density before induction on the anti EpEX-scFv production is presented in Fig. 5F. Based on the high P value (0.8061) of the coefficient cell density before induction and IPTG concentration (Table3), there is no significant interaction between these two parameters. Figure 5F reveals that the production of the anti EpEX-scFv protein will be improved by increasing OD600 while changes in the value of the IPTG concentration has no effect on the anti EpEX-scFv expression level.

Based on the obtained results, the model predicted that the maximum anti EpEX-scFv production could be achieved by the following optimal culture conditions: IPTG concentration of 0.8 mM, cell density before induction (OD600) of 0.8, the post-induction temperature of 37 °C, and post-induction time of 24 h. Finally, to validate the model, the expression of anti EpEX-scFv was carried out using the optimum conditions predicted by the model (in three repeats). As a result, 197.33 µg/mL of the recombinant anti EpEX-scFv protein was achieved, which was very close to the predicted value (181.07 µg/mL; Fig. 6). So, the suitability and accuracy of the CCD model were fully confirmed for recombinant anti EpEX-scFv production.
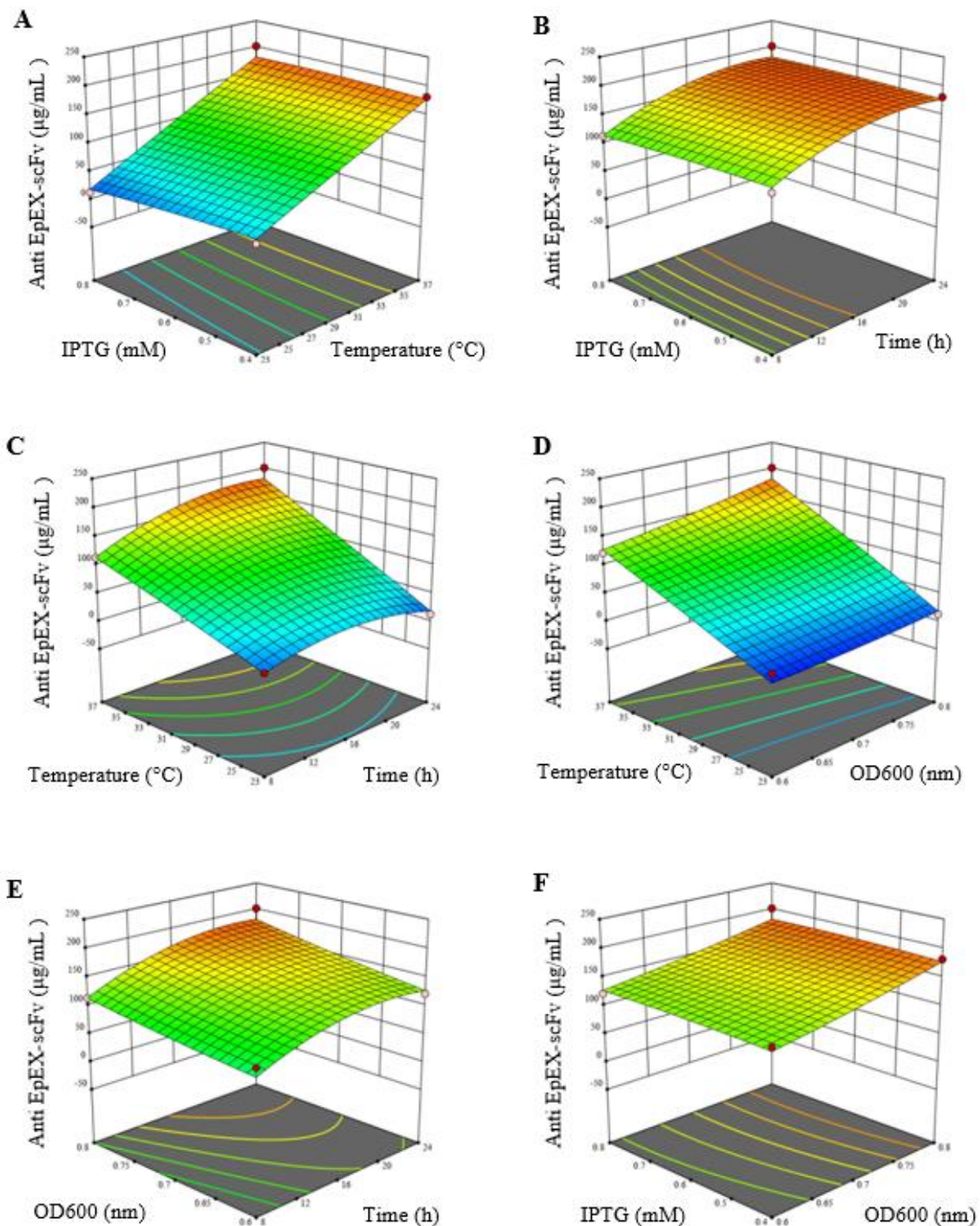
Fig. 5. Response surface plots to illustrate the interaction of experimental variables on the anti EpEX-scFv expression level using the RSM-CCD methodology. Interactive effects of (A) IPTG concentration and induction temperature; (B) IPTG concentration and post-induction time, (C) induction temperature and post-induction time, (D) induction temperature and cell density (OD600), (E) cell density (OD600) and post-induction time, (F) IPTG concentration and cell density (OD600) on anti EpEX-scFv expression level. IPTG, Isopropyl-b-D-thiogalactopyranoside; RSM-CCD, response surface methodology-central composite design; OD, optical density.
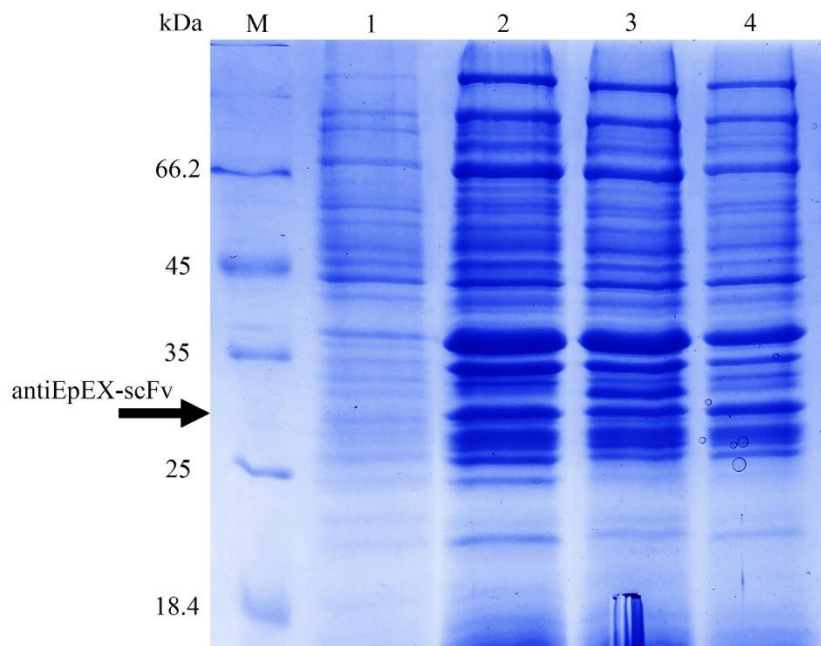
Fig. 6. SDS-PAGE analysis for characterization of the recombinant anti EpEX-scFv protein in the optimum expression conditions. Lane M, unstained protein molecular weight marker (14.4-116 kDa); lane 1, uninduced bacterial lysate; lanes 2-4, three repeats of the experiments in the optimum conditions in M9 minimal medium. The arrow indicates the band of the anti EpEX-scFv protein with the expected size of ~ 29 kDa. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

DISCUSSION

As the cost-effectiveness and reproducibility of the production process of recombinant proteins are the main concerns of biopharmaceutical industries, chemically defined-minimal media as perfect solutions can be used to achieve these two targets (21). In this study, the design of the experiments approach was employed for investigating the effective parameters on the recombinant anti EpEX-scFv production and achieving the maximum production yield in a chemically defined-minimal medium. For this purpose, a total of 30 experiments were designed to assess the interactions of four main influential factors (cell density before induction, IPTG concentration, post-induction temperature, and post-induction time) on the recombinant anti EpEX-scFv production in *E. coli* BW25113 (DE3) by employing RSM/CCD methodology. Finally, a quadratic model (equation 1) was suggested by Design Expert software based on the experimental results. The developed model demonstrated that the four variables including post-induction time, cell density before induction, post-induction temperature, and IPTG concentration have significant effects on

the anti EpEX-scFv production (Table 3). 3D response surfaces (Fig. 5) are illustrating the anti EpEX-scFv production as a function of the independent variables' interaction. Statistical analysis and 3D plots indicated that the optimum culture conditions for high-yield anti EpEX-scFv production could be achieved by the following optimal culture conditions: cell density before induction at 0.8 nm, IPTG concentration of 0.8 mM, and 37 °C for 24 h. Moreover, the experimental observation at optimum conditions (197 µg/mL) was in good agreement with the prediction of the proposed model (181 µg/mL).

The post-induction temperature and post-induction time are two important factors that have effective roles in the expression level of the recombinant proteins (14,22). According to the results, a combined effect of post-induction temperature and post-induction time could lead to the highest anti EpEX-scFv expression level. During the experiments, anti EpEX-scFv in the form of inclusion bodies was produced at a temperature above 30 °C but when the temperature was shifted down, the total expression level was significantly decreased. Consistently, Shafiee and colleagues (23), Akbari and coworkers (24), and Volontè *et al.* (25) in different investigations mentioned that

the highest level of the recombinant protein expression could be achieved at 37 °C. Furthermore, a long incubation time was shown to be critical for the optimal expression of anti-tumor necrosis factor α scFv in *E.coli* (26). In contrast, for some recombinant proteins, low temperature and post-induction time (< 8 h) were appropriate (27). Therefore, the optimum post-induction time and temperature depend on the type of protein and the host. Cell density before induction is also important in the expression level of recombinant proteins (28). The results of this study demonstrated that the induction of recombinant anti EpEX-scFv in the middle and close to the late-log phase could lead to a higher expression of the protein. In this phase, most recombinant bacteria are growing rapidly, and cells are in an ideal situation for the recombinant-protein expression. Galloway *et al.* have also reported that induction at the late-log phase could lead to a high level of the recombinant ACF64 protein expression (29). The determination of the optimal inducer concentration is also necessary because expression of the protein may not occur at low concentrations and on the other hand, high concentrations of inducer maybe toxic for the bacteria (30). Therefore, a reasonable range of IPTG concentration was determined for the experiments. The results showed a significant correlation between IPTG concentration and the production of anti EpEX-scFv.

CONCLUSION

In conclusion, in this study culture, conditions for the recombinant anti EpEX-scFv production in *E. coli* BW25113 (DE3) were successfully optimized by RSM and it should be emphasized that the medium was a chemically defined one. A combination of optimized culture conditions and using this kind of medium would be a perfect solution for large-scale fermentation, where the process is requested to be economic and reproducible. To our knowledge, this is the first report showing this combination that can be used for industrial production of the recombinant anti EpEX-scFv protein.

Conflict of interest statement
The authors declared no conflict of interest in this study.

Authors' contribution
A. Hashemi has made substantial contributions to conception and design. A. Behravan carried out the experiments and analyzed the data. A. Behravan wrote the manuscript. A. Hashemi reviewed and edited the article for spelling, grammar, and intellectual content; organized and supervised the whole project; and provided the facilities and materials required for the project. Both authors agreed to be responsible for the content of the work.

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