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RESEARCH ARTICLE

Enhancement of extracellular bispecific anti-MUC1 nanobody expression in *E. coli* BL21 (DE3) by optimization of temperature and carbon sources through an autoinduction condition

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Abstract

Escherichia coli is one of the most suitable hosts for production of antibodies and antibody fragments. Antibody fragment secretion to the culture medium improves product purity in cell culture and diminishes downstream costs. In this study, *E. coli* strain BL21 (DE3) harboring gene encoding bispecific anti-MUC1 nanobody was selected, and the autoinduction methodology for expression of bispecific anti-MUC1 nanobody was investigated. Due to the replacement of IPTG by lactose as inducer, less impurity and toxicity in the final product were observed. To increase both intracellular and extracellular nanobody production, initially, the experiments were performed for the key factors including temperature and duration of protein expression. The highest amount of nanobody was produced after 21 h at 33°C. The effect of different carbon sources, glycerol, glucose, lactose, and glycine as a medium additive at optimum temperature and time were also assessed by using response surface methodology. The optimized concentrations of carbon sources were obtained as 0.75% (w/v), 0.03% (w/v), 0.1% (w/v), and 0.75% (w/v) for glycerol, glucose, lactose, and glycine, respectively. Finally, the production of nanobody in 2 L fermenter under the optimized autoinduction conditions was evaluated. The results show that the total titer of 87.66 μg/mL anti-MUC1 nanobody, which is approximately seven times more than the total titer of nanobody produced in LB culture medium, is 12.23 μg/L .

KEYWORDS

autoinduction, bispecific anti-MUC1 nanobody, *E. coli* BL21 (DE3), extracellular protein expression, medium optimization

1 | INTRODUCTION

Escherichia coli has been used as a host for production of recombinant proteins, including antibodies and antibody fragments, as it is fast growing, easy to manipulate, and genetically stable in large cultures. However, the drawbacks in application of *E. coli* in large scales are intracellular production of recombinant proteins [1]. Therefore, genetic manipulation of these bacteria besides the optimization of culture media and operation conditions might improve

Abbreviation: LB, Luria–Bertani.

extracellular production. If the whole protein was produced extracellularly into the culture medium, the product purity from cell culture increases, downstream processing requirements decrease, and also the overall cost diminishes [2]. It has been reported that the expression of recombinant proteins under the control of lac operon induced by IPTG leads to low expression of recombinant proteins. According to the previous studies, due to the high cost and toxicity of IPTG, complex operating procedure, and nonuniform protein expression pattern, the use of lactose as an inducer for expression of recombinant proteins is preferable for overproduction of recombinant proteins [3]. By using lactose as inducer more soluble protein has been produced. The secretion of protein from bacteria occurred through highly specialized systems, in which the proteins could be translocated in either one or two steps from the cytosol to the extracellular medium. Based on previous successes, researchers continue to increase the secretion efficiency and titer through these systems to make them feasible for industrial production. These approaches include the modification of secretion tags required for secretion of the recombinant protein, development of methods for rapid screening, or selecting clones with higher titer or efficiency, and the use of genetic engineering for improving reliability and robustness of high titer secretion [4]. Also, the modification of the culture medium by suitable substances, such as glycine, SDS, sucrose, Tween 80, and TritonX-100, could be useful in extracellular production of proteins in bacteria [5, 6]. The optimization of carbon sources shows a great impact on the overproduction of recombinant proteins [7]. Finding an optimum culture media for extracellular production of proteins which are produced in *E. coli* has always been a concern. The study aimed to enhance the production of bispecific anti-MUC1 nanobody intracellularly and extracellularly from *E. coli* BL21 (DE3). Therefore, in an autoinduction medium, the temperature, duration of induction, concentration of the carbon sources, and glycine as an additive were optimized to obtain the maximum quantity of nanobody. Based on the detailed literature review, it was the first study that considered this method for the production of nanobodies.

PRACTICAL APPLICATION

The result obtained in fermenter shows the concentration of 74.43 μg/mL, which is approximately seven times higher than nanobody produced in LB culture medium 12.23 μg/L in shake flask under the same conditions.

2 | MATERIALS AND METHODS

2.1 | Bacterial strain and plasmid

Recombinant *E. coli* strain BL21 (DE3) harboring bispecific anti-MUC1 nanobody gene, which was previously constructed (Motamed Cancer Institute, Tehran, Iran) with a molecular weight of 35 kDa, was used as a model for the expression of a recombinant nanobody. Stock cultures of this strain were maintained in 30% glycerol (v/v) at −80°C. The *E. coli* BL21 DE3 (EMD-Millipore, MA, USA) is an *E. coli* cell suitable for transformation and high-level protein expression using a T7 RNA polymerase-IPTG induction system. The pET-32 Ek/LIC vector (EMD-Millipore) is commercially available for rapid, directional cloning of PCR-amplified DNA for high-level expression of polypeptides fused with the 109aa Trx•Tag^{TM} thioredoxin protein [8].

2.2 | Chemicals

Chemicals and reagents used in this study for the optimization of the culture media were obtained from Sigma (St. Louis, MO, USA), Fitzgerald (GA, USA), Bio-Rad (CA, USA), Hi-Media (Mumbai, India), Padtan Elm (Tehran, Iran), and LB Broth (NJ, Merck). SDS-PAGE reagents were purchased from Bio-Rad, and protein molecular weight markers were obtained from SinaClon (Tehran, Iran). Antihuman IgM-HRP and antimouse IgG-HRP were obtained from Cito Matin Gen (Tehran, Iran). Besides, antibiotic, ampicillin (100 μg/mL), was obtained from Abidi Pharmaceutical Co. (Tehran, Iran).

2.3 | Culture media

Two different culture mediums were investigated in this study. (1) Luria–Bertani medium (LB: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl), which was the maintenance medium for recombinant *E.coli*. (2) Autoinduction medium.

2.4 | Bispecific anti-MUC1 nanobody

Anti-MUC1 nanobody coding sequence was kindly provided by medical biotechnology group, Faculty of Medical Sciences, Tarbiat Modares University [9]. The sequence was then engineered based on modeling programs to improve the affinity of nanobody to the MUC1 tandem repeats. Bispecific anti-MUC1 nanobody cassette was designed and synthesized in our group, previously. The detailed steps of construction of recombinant *E.coli* harboring above-mentioned nanobody was reported in our previous works [10]. Briefly, a stII signal peptide coding sequence was introduced to the 3′ end of the bispecific anti-MUC1 nanobody coding sequence for periplasmic and extracellular localization of nanobody. Moreover, a six constitutive histidine tag coding sequence was introduced at the 5′ end of the bispecific anti-MUC1 nanobody coding sequence for purification by Ni-NTA affinity chromatography. The full-length sequence was then codon optimized for expression in *E. coli* and

synthesized (Cinnagen Inc, Tehran, Iran). The codon-optimized sequence was amplified with PCR, then purified and concentrated with a gel extraction kit (Qiagen, Düsseldorf, Germany). Subsequently, amplicons were cloned into the LIC site of the pET-32 Ek/LIC vector (EMD-Millipore) according to the manufacturer's instructions and used to transform NovaBlue GigaSingles^{TM} competent cells (EMD-Millipore). The recombinant clones were then randomly confirmed using colony PCR and sequencing. Recovered plasmids from positive colonies were used to transform protein expression strain *E. coli* BL21 DE3 (EMD-Millipore) competent cells.

3 | INOCULUM PREPARATION

3.1 | LB media

For inoculum preparation, 5 mL of LB medium containing ampicillin (100 μg/mL) was inoculated using a single colony from agar plates of the *E. coli* strain BL21 (DE3) and incubated at 37°C, 180 rpm for 12 h.

3.2 | Autoinduction media

The recipes for the preparation of the autoinduction medium was introduced by Studier [11]. Briefly, the medium was prepared using deionized water and then sterilized by 0.2, 0.5, and 0.75 μm filters. Solution (1) containing 134 g NH_4Cl, 170 g KH_2PO_4, 35.5 g Na_2SO_4, and 209 g Na_2HPO_4 per liter was prepared fresh and heat sterilized. The second solution, which is the carbon sources of the media, was prepared by the combination of 250 g glycerol 0.5% w/v, 5 g glucose 0.05% w/v, and 100 g α-lactose 0.2% w/v per liter, the stock of each carbon source was prepared separately and then mixed. The constituents were added in the specified order, stirred until dissolved, and the final volume was adjusted to 1 L. This solution was also prepared fresh and sterilized [11, 12]. The 1000 × trace metals solution was prepared by 100 mL aliquot containing 50 mL of 0.1 M $FeCl_3 \cdot 6H_2O$ dissolved in 0.1 M HCl, 2 mL of 1 M $CaCl_2$, 1 mL of 1 M $MnCl_2 \cdot 4H_2O$, 1 mL of 1 M $ZnSO_4 \cdot 7H_2O$, 1 mL of 0.2 M $CoCl_2 \cdot 6H_2O$, 2 mL of 0.1 M $CuCl_2 \cdot 2H_2O$, 1 mL of 0.2 M $NiCl_2 \cdot 6H_2O$, 2 mL of 0.1 M $Na_2MoO_4 \cdot 5H_2O$, 2 mL of 0.1 M $Na_2SeO_3 \cdot 5H_2O$, and 2 mL of 0.1 M H_3BO_3. All stock solutions of individual metals except acidified $FeCl_3 \cdot 6H_2O$ were heat sterilized and stored at 25°C until use. The trace element solution was wrapped in aluminum foil and stored at 25°C [11].

4 | NANOBODY PRODUCTION IN SHAKE FLASK

Glycerol stocks of recombinant *E. coli* BL21 (DE3) were streaked on LB solid medium supplemented with corresponding antibiotics and incubated overnight at 37°C. A single colony was selected and inoculated in 4 mL LB medium for preparing seed culture. Then, it was grown at 37°C and kept in a shaker at 200 rpm for 4 h until it reached OD600 of 2–3. Subsequently, 500 μL of seed culture was inoculated into 250 mL flask containing 30 mL LB or autoinduction medium and cultured under the same condition as described above. For induction, when the optical density at 600 nm of culture reached 0.9, the IPTG was added to a final concentration of 1.0 mM to induce the expression of the target gene for 22 h.

5 | NANOBODY PRODUCTION IN THE FERMENTER

To express nanobody in the fermenter, 100 mL of inoculum (OD_{600}∼2) was inoculated into a 2 L fermenter (Zist Farayand Sanat Saba, Iran). Then, the *E. coli* BL21(DE3) harboring the bispecific anti-MUC1 nanobody gene was activated at an agitation rate of 400 rpm and an aeration rate of 1 vvm, at pH 7 for 12 and 14 hr for LB and autoinduction medium, respectively. Polypropylene glycol (PPG) was used as antifoam, and 2 M NaOH and 2 M HCl were used to adjust the pH of culture.

5.1 | Analyses of expressed anti-MUC1 nanobody

At the end of each batch, the culture samples were centrifuged at 8000 rpm, for 10 min at 4°C. Then, the lysis buffer, which contained 25 mM EDTA, 30 Mm Tris, and 200 mM NaCl, was added to the pellets and the suspension was sonicated 15 times each 20 sec and the time interval between each time was 60 sec, 1-min pulse, on the ice to avoiding the nanobody disruption. Finally, the lysed cells were centrifuged at 8000 rpm for 20 min 4°C, and the supernatant of that was collected for measurement of the amount of produced nanobody by ELISA assay. A total amount of 0.1 g of harvested cells were suspended in 100 μL of loading buffer containing 2% mercaptoethanol, heated for 5 min, and centrifuged at 10 000 rpm for 30 s. Subsequently, the 12% polyacrylamide gel was used to identify the extracellular and intracellular secretion of nanobody [13]. Cell growth was evaluated by measurement of the optical density of the cultures using spectrophotometer (Cary 100, Varian, CA, USA) at 600 nm.

6 | ELISA ASSAY

The binding activity of bispecific anti-MUC1 nanobody, which has His-tag, against the ca15-3 protein, was evaluated using ELISA. Briefly, a 96-well microplate was coated with 50 μL of CA15-3 protein (Fitzgerald) concentration of 1000 U/mL in a coating buffer (sodium carbonate at pH 6.9) then microplate was covered with Parafilm and placed

TABLE 1 The production of biomass at different temperatures for two sets of experiments (14 and 21 h) to find an optimized temperature

Temperature (°C)	Biomass (g/L) (14 h)	(21 h)
20	5.71	6.57
25	7.60	6.74
27	6.78	7.02
30	7.41	7.70
33	8.08	9.31
37	6.33	8.87

overnight at 4°C. After washing four times with PBS containing Tween 20, the wells were blocked with 5% skim milk in PBST and incubated at 37°C for 2 h. Then, 100 μL of the samples after sonication containing bispecific anti-MUC1 nanobody were transferred into wells in separate rows and diluted in series. The plate was placed overnight at 4°C. After washing with PBST, 100 μL of a 1 of 500 dilution of HRP-conjugated anti-His antibody was added and incubated for 1 h at 37°C. The reaction was developed using 100 μL of 3, 3′,5,5′-tetramethylbenzidine chromogenic (TMB) substrate. The reaction was stopped with 2 M H_2SO_4 after 15 min, and optical density was measured at 450 nm using an automicroplate reader (Awareness Technology Stat Fax 2100, MN, USA). Each experiment was performed in triplicate.

6.1 | Quantification of bispecific anti-MUC1 nanobody

Following confirmation of the specificity of the interaction between bispecific anti-MUC1 nanobody and CA15-3 protein using ELISA, quantification of this nanobody was investigated. For this purpose, we used purified bispecific anti-MUC1 nanobody as the standard sample, the concentration was determined using UV-Vis spectrophotometer at 280 nm. Thus, we selected different concentrations between 1 and 1000 μg/mL to generate the standard curve for bispecific anti-MUC1nanobody. We used the standard curve for finding the concentration of bispecific anti-MUC1 nanobody in our experiments.

7 | RESULTS

7.1 | Optimization of temperature and time

Some preliminary experiments were designed to obtain the optimum time and temperature for the maximum production of biomass and nanobody in the LB medium. For this purpose, two sets of experiments were performed at 20, 25, 27, 30, 33, 37°C for 14 and 21 h. The results of biomass and nanobody production are presented in Table 1 and Figure 1, respectively.

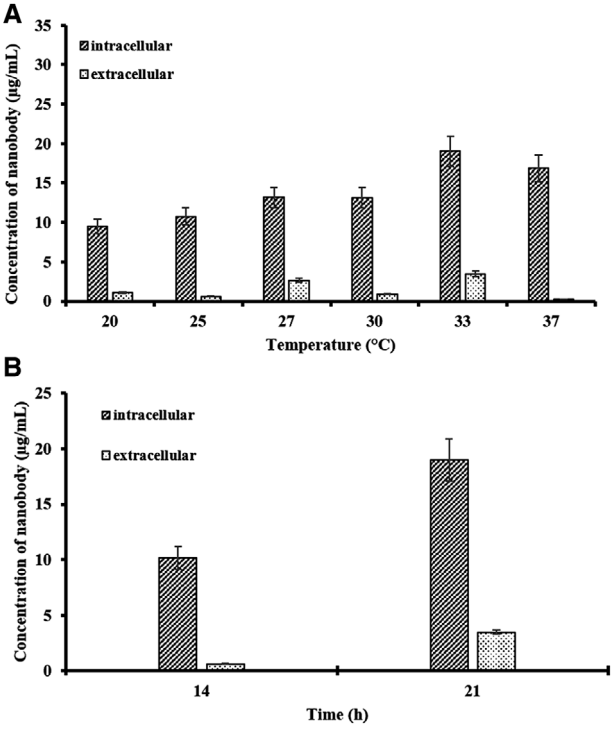

FIGURE 1 Standard curve of bispecific anti-MUC1 nanobody as determind by ELISA

Based on the results presented in Table 1, by increasing temperature for both 14 and 21 h, the biomass increases; but, for $T > 33$°C, it decreased sharply after 14 h. The results in Figure 1A and B have demonstrated that the production of both intracellular and extracellular nanobody was higher at 33°C after 21 h. Under this condition, the amount of nanobody produced intracellularly and extracellularly was 9.01 and 3.46 μg/mL, respectively. Moreover, by enhancement, the time of culture, production of nanobody was increased (Figure 2). These results confirm that nanobody production is growth-related and therefore $T = 33$°C and $t = 21$ h were selected for further optimization.

7.2 | Optimization of carbon sources

The response surface methodology was used to evaluate the relationship between a group of controlled variables and their effects. Using this optimization methodology, the concentration of various carbon sources, including glycerol, glucose, lactose, and also glycine, as an additive, in the medium were selected. Thirty different experiments were conducted according to Tables 2 and 3.

Among the possible regression models, the modified cubic model was employed for the analysis of the results. Equations 1 to 4 represent the dependency of concentration of glycerol, glucose, lactose, and glycine on biomass, extracellular, intracellular and total amount of nanobody production,

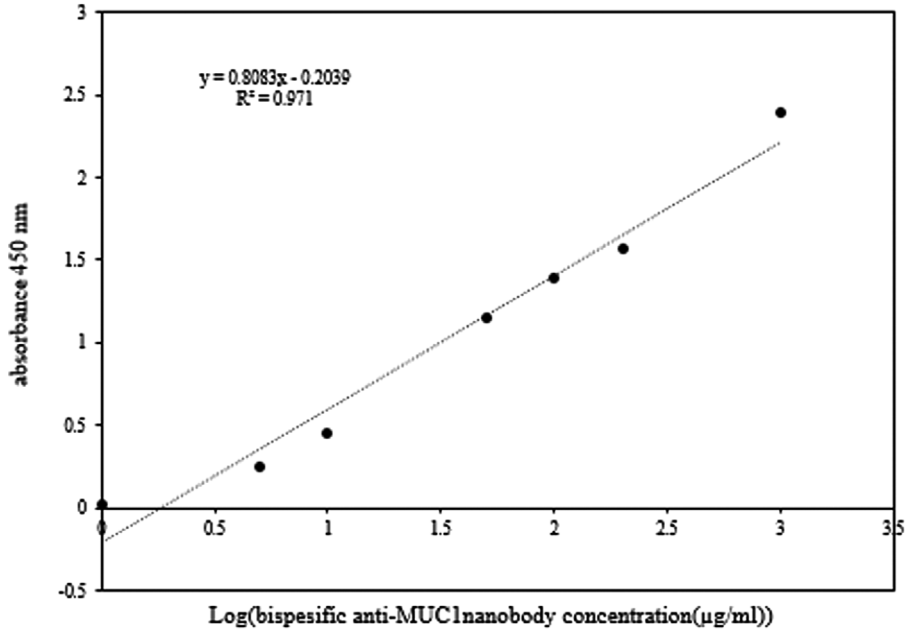

FIGURE 2 (A) The effect of different temperatures on intracellular and extracellular production of nanobody after 21 h and (B) the effect of time on the production of intracellular and extracellular production of the nanobody at 33°C

respectively:

$$\begin{aligned}\text{Biomass} = {} & +7.77 + 2.15 * A + 0.025 * B + 0.26 * C \\ & - 2.26 * D - 0.39 * A * B + 0.32 * A * C \\ & - 0.92 * A * D + 0.27 * B * C \\ & - 0.29 * C * D - 0.65 * A^2 - 1.02 * B^2 \\ & - 0.58 * C^2 + 0.69 * D^2 \end{aligned} \quad (1)$$

Extracellular nanobody

$$\begin{aligned} = {} & +2.74 + 0.046 * A - 0.80 * B - 0.38 * C \\ & - 0.35 * D - 0.61 * A * B - 0.25 * A * C \\ & - 0.43 * A * D + 0.14 * B * C + 0.68 * B * D \\ & + 0.074 * C * D + 0.26 * A^2 + 0.21 * B^2 \\ & - 0.045 * D^2 + 0.12 * A * B * C \\ & + 0.71 * A * B * D + 0.29 * A * C * D \\ & - 0.098 * B * C * D + 0.53 * A^2 * C \\ & + 0.50 * A * B^2 + 0.19 * B^3 + 0.043 * D^3 \end{aligned} \quad (2)$$

Intracellular nanobody

$$\begin{aligned} = {} & +32.62 - 3.71 * A - 0.41 * B + 13.78 * C \\ & + 5.09 * D + 1.33 * A * B - 10.10 * A * C \\ & + 3.99 * A * D - 2.60 * B * C - 9.89 * B * D \\ & + 0.24 * C * D - 5.20 * A^2 + 6.55 * B^2 \\ & + 2.75 * C^2 + 9.43 * D^2 - 3.47 * A * B * D \\ & - 8.16 * A * C * D - 4.86 * A^2 * B \\ & - 15.29 * A^2 * C - 5.73 * A * B^2 \end{aligned} \quad (3)$$

TABLE 2 Variables used in CCD response surface design

Parameter	Unit	−1 level	+1 level	0	−alpha	+alpha
Glycerol (A)	(%w/w)	0.25	0.75	0.5	0	1
Glucose (B)	(%w/w)	0.02	0.07	0.05	0	0.1
Lactose (C)	(%w/w)	0.1	0.3	0.2	0	0.4
Glycine (D)	(%w/w)	0.37	1.12	0.75	0	1.5

Total nanobody

$$\begin{aligned} = {} & +38.64 - 3.66 * A - 3.90 * B + 13.40 * C \\ & + 5.01 * D + 0.99 * A * B - 10.09 * A * C \\ & + 3.82 * A * D - 1.83 * B * C - 8.58 * B * D \\ & + 0.94 * C * D - 5.25 * A^2 + 6.46 * B^2 \\ & + 9.08 * D^2 - 7.24 * A * C * D \\ & + 4.56 * B * C * D \\ & - 14.50 * A^2 * C - 4.60 * A * B^2 \end{aligned} \quad (4)$$

The effect of glycerol, glucose, lactose, and glycine concentration on the extracellular production of nanobody is shown

TABLE 3 CCD experimental runs and corresponding responses

Run	Glycerol (%w/w)	Glucose (%w/w)	Lactose (%w/w)	Glycine (%w/w)	Biomass (g/L)	Extracellular nanobody (μg/mL)	Intracellular nanobody (μg/mL)	Total nanobody (μg/mL)
1	0.75	0.07	0.1	1.12	5.04	2.81	39.8	42.6
2	0.5	0.05	0.2	0.75	8	2.57	15.7	18.3
3	1.00	0.05	0.20	0.75	4.85	3.80	1.54	5.35
4	0.25	0.07	0.30	1.12	2.56	2.91	56.0	58.9
5	0.25	0.02	0.10	0.37	4.96	1.90	56.0	57.9
6	0.5	0.00	0.20	0.75	2.39	3.62	56.7	60.4
7	0.25	0.07	0.30	0.37	5.66	3.20	56.0	59.2
8	0.5	0.05	0.20	1.50	3.26	2.14	78.9	81.0
9	0.5	0.05	0.00	0.75	3.96	3.32	13.2	16.5
10	0.25	0.075	0.10	0.37	6.03	1.89	56.0	57.9
11	0.75	0.075	0.10	0.37	9.59	1.89	56.2	58.1
12	0.5	0.05	0.20	0.00	15.2	2.87	56.0	58.9
13	0.5	0.05	0.20	0.75	7.72	2.82	36.0	38.8
14	0.5	0.05	0.20	0.75	7.72	2.82	36.0	38.8
15	0.25	0.02	0.30	1.12	2.94	2.97	93.5	96.5
16	0.75	0.07	0.30	0.37	12.3	1.84	21.3	23.1
17	0.25	0.07	0.10	1.12	2.68	2.54	33.5	36.1
18	0.75	0.02	0.30	1.12	5.15	3.00	35.3	38.3
19	0.25	0.02	0.30	0.37	4.18	3.08	56.8	59.8
20	0	0.05	0.20	0.75	1.44	3.61	16.3	19.9
21	0.5	0.10	0.20	0.75	4.14	3.43	55.1	58.5
22	0.25	0.02	0.10	1.12	3.17	2.61	48.1	50.7
23	0.75	0.02	0.10	1.12	10.0	2.65	93.0	95.7
24	0.5	0.05	0.40	0.75	6.06	1.79	68.3	70.1
25	0.75	0.02	0.10	0.37	11.8	10.8	9.86	20.7
26	0.5	0.05	0.20	0.75	7.72	2.82	36.0	38.8
27	0.5	0.05	0.20	0.75	7.72	2.82	36.0	38.8
28	0.75	0.02	0.30	0.37	13.5	6.15	30.0	36.2
29	0.5	0.05	0.20	0.75	7.72	2.82	36.0	38.8
30	0.75	0.07	0.30	1.12	8.57	10.6	19.4	30.0

in Figure 3. Each parameters has a direct impact on extracellular production. The effect of two factors, glucose and glycerol, at a constant concentration of 0.38% w/v glycine and 0.1% w/v lactose, were investigated and the results are shown in Figure 1A. At the constant concentration of glucose, the results revealed that by increasing glycerol concentration, the amount of extracellular nanobody is enhanced. Generally, the maximum amount of extracellular nanobody has been produced at the lowest concentration of glucose and at the highest concentration of glycerol [11, 12]. Technically, in high concentrations of glucose, catabolic repression occurs and, the presence of excessive glucose in the medium leads to the production of unwanted byproducts like acetate. Therefore, it causes the acidity of the culture medium and lower production of the target protein [2]. On the other hand, glycerol has shown positive effects on protein expression. Based on the results shown in Figure 3, at constant concentration of lactose, enhancement of the glycerol concentration increased the amount of extracellular nanobody.

The effect of glucose, glycerol, and lactose concentration on the intracellular amount of nanobody has shown the same results as extracellular. However, different concentrations of glycine have impacted the intracellular production of nanobody (Figure 3A-C). The highest concentration of glycine (1.13 % w/v) in the media has the most desirable impact on the intracellular nanobody. Thirteen percent of nanobody building blocks consists of glycine. So, addition of glycine to the culture medium both reduces the metabolic burden on bacteria and acts as a precursor for the production of the target protein [5, 6]. The results from total amount

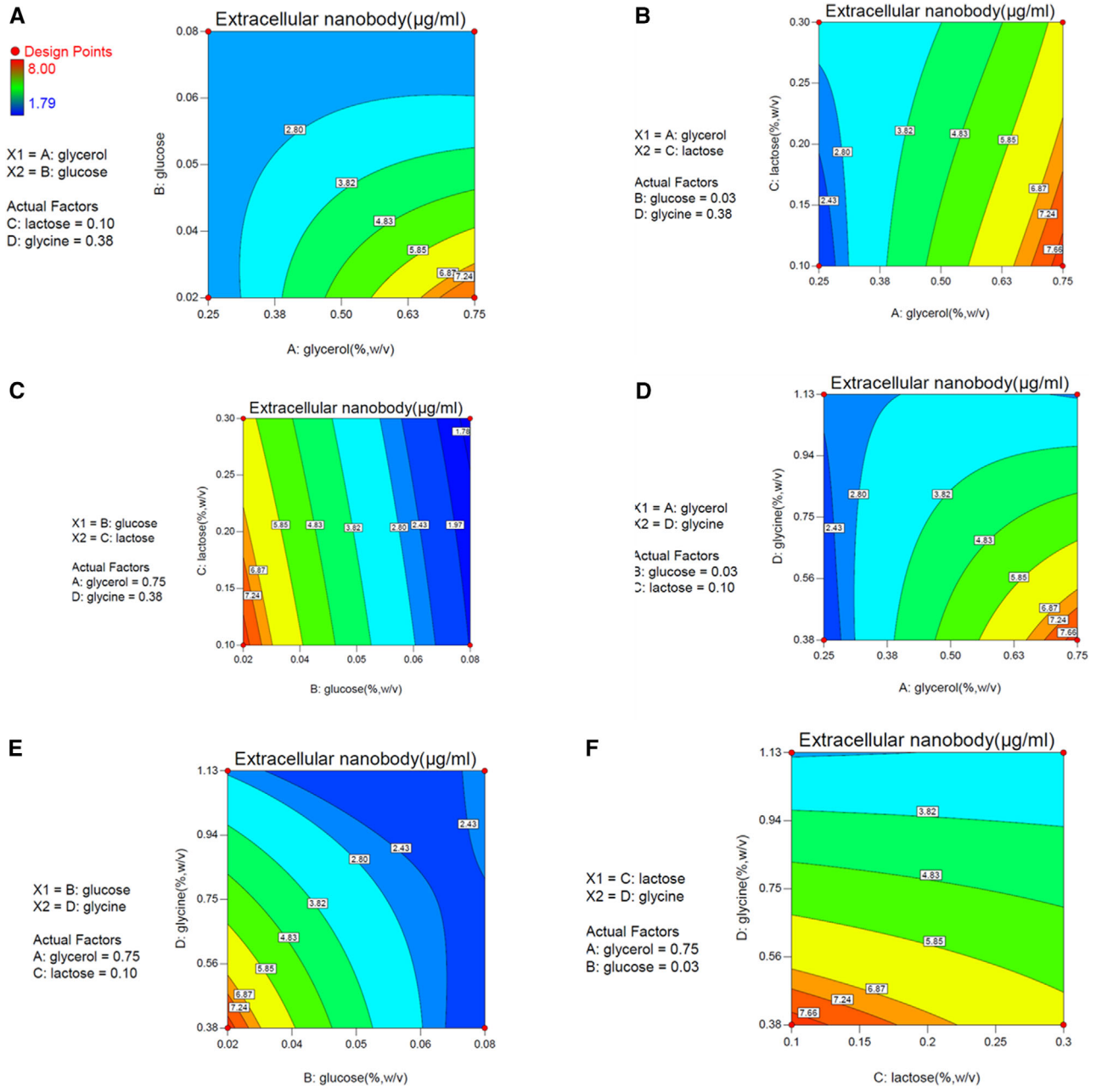

FIGURE 3 The contour plots for extracellular production of nanobody: (A) the effect of glucose and glycerol, (B) the effect of lactose and glycerol, (C) the effect of lactose and glucose, (D) the effect of glycine and glycerol, (E) the effect of glycine and glucose, and (F) the effect of glycine and lactose (other carbon sources were kept constant for each experiment)

TABLE 4 Predicted models of the responses with statistical analysis

Response	R^2	Adj. R^2	Adeq. precision	C.V.%	*p*-value
Extracellular nanobody (µg/mL)	0.99	0.97	38.9	6.00	<0.0001
Intracellular nanobody (µg/mL)	0.89	0.70	8.27	28.9	<0.0088
Total nanobody (µg/mL)	0.87	0.69	7.99	26.1	<0.0041
Biomass (g/L)	0.95	0.91	21.4	14.8	<0.0001

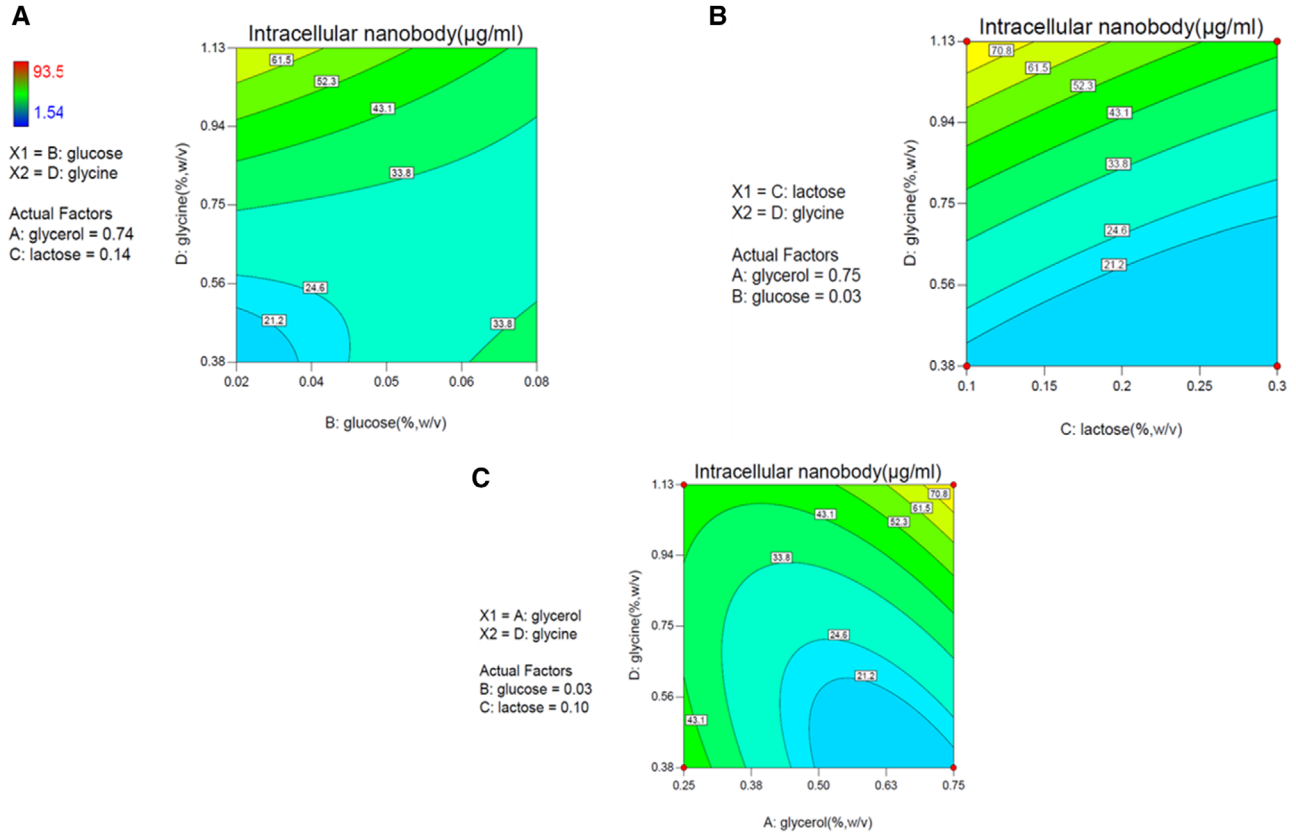

FIGURE 4 The contour plots for intracellular production of nanobody: (A) the effect of glycerol and glycine, (B) the effect of lactose and glycine, (C) the effect of glycine and glycerol (other carbon sources were kept constant for each experiment)

TABLE 5 Point prediction of the responses at the optimal conditions response

Response	Prediction	SE Mean[a]	95% CI low[b]	95% CI high[c]	SE Pred.[d]	95% PI low[e]	95% PI high[f]
Extracellular nanobody (µg/mL)	5.40	-	5.11	5.71	-	4.89	5.93
Intracellular nanobody (µg/mL)	39.50	8.25	21.13	57.88	15.03	6.02	72.99
Total nanobody (µg/mL)	43.16	7.86	26.05	60.28	14.57	11.41	74.92
Biomass (g/L)	7.77	0.59	6.53	9.03	1.13	5.38	10.18

[a]Standard errors of the mean.
[b]Confidence interval of the parameters (the minimum amount of nanobody that should be and the reliable level is selected 95%).
[c]Confidence interval of the parameters (the maximum amount of nanobody that should be and the reliable level is selected 95%).
[d]Standard error which is predicted.
[e]Predicted interval of parameters (the lower boundary for the next new observation for the amount of nanobody that will fall within this and the upper boundary and selected to be 95% as a reliable level).
[f]Predicted interval of parameters (the upper boundary for the next new observation for the amount of nanobody that will fall within this and the lower boundary and selected to be 95% as a reliable level).

of nanobody (Figure 4), the cytoplasm, and periplasm membrane and secreted into the culture media, have revealed reasonable consistency with the results which have been obtained for both extracellular and intracellular nanobody. To illustrate this, a high concentration of glucose causes catabolic repression, as well as the increase of acidity of the culture medium. Therefore, low concentration (0.02% w/v) was selected for glucose in the medium, and the optimum concentrations of 0.75% and 0.1% (w/v) for glycerol and lactose were used in the induction of genes expression, respectively. Figure 4A and B shows the effect of glucose and glycine in total nanobody production. These results have revealed that the production of nanobody improved by increasing glycine concentration and by decreasing glucose concentration. Moreover, Figure 4C and D shows contours and 3D plots for the effect of both glucose and lactose on total nanobody production when both, glycine and glycerol, were kept constant at optimal conditions. Also, the effects of both glucose and glycerol on biomass were investigated while lactose and glycine were kept at a constant concentration (Figure 4). By increasing the concentration of glucose to approximately 0.05% (w/v), the growth rate has reached the highest level, on the other hand, exces-

sive glucose in the medium increases acidity. Subsequently, the growth rate has declined [6, 7, 11]. Figure 4F demonstrates the effect of both glycerol and glycine concentrations. At constant concentrations of glycerol, the growth increases as glycine concentration decreases. As it is mentioned above, the presence of glycine in the culture medium improves bacterial growth. Optimization is about finding the best possible solution or method from a set of applicable solutions [11]. In this study, to obtain optimal condition in which the highest amount of nanobody was produced, in the design experiment software, biomass and intracellular production of nanobody were selected to be in range, which were from 1.548 to 93.596 and 1.44 to 15.23, respectively, and also extracellular and total production of nanobody maximum amount of possible were determined. Table 4 presents the summary of the software predicted values for the maximum production of nanobody. Based on the statistical models presented here, the optimized concentrations for glycerol, glucose, lactose, and glycine in culture media were obtained at 0.75% w/v, 0.03% w/v, 0.1% w/v, and 0.75% w/v, respectively. The estimated values for the production of nanobody and biomass in the optimized medium obtained were 6.20 μg/mL for extracellular production of nanobody, 37.53 μg/mL for intracellular production of nanobody, 43.73 μg/mL total production of nanobody, and 6.86 g/L for biomass. To confirm the model prediction and certify the presence of an optimum point, validation experiments were performed at the optimal point. For each response, confirmation tests were done in triplicate. According to the results presented in Table 5, the estimated values from the design of experiment software and the values obtained from the experiments are in good agreement. This implies that the models for the responses are in good agreement with the conditions prevailing in the experiment. The reliable level selected in the Table is 95%.

8 | NANOBODY PRODUCTION IN THE FERMENTER

The results obtained under the optimum condition from the shake-flask cultivation for the production of nanobody were also investigated in the fermenter. Production of nanobody was performed in two different LB cultures and autoinduction media under controlled conditions of temperature, pH, and aeration rate in a 2-L fermenter. Finally, the titer of produced nanobody was compared in these two media. For the LB medium in the fermenter, in which the induction was carried out by 1 mM IPTG, operating temperature before induction was set at 37°C and after induction was set at 27°C. As a result, cell density and yield of intracellular produced nanobody were 7.15 g/L and 12.23 μg/mL, respectively. On the other hand, the results of nanobody production in the autoinduction culture medium were considerable. The optimized autoinduction culture condition, which was obtained in shake flask, was investigated in the fermenter. The process continued until the steady bacterial growth rate was observed (end of the log phase). Consequently, both intracellularly (78.43 μg/mL) and extracellularly (9.23 μg/mL) and the quantity of total nanobody (87.66 μg/mL) and biomass were approximately twofold higher than shake flask cultivation. The higher yield of nanobody in the fermenter is due to the precise control of environmental conditions during nanobody production. Figure 5A and B shows that the cell density and the yield of nanobody production in an autoinduction medium increased compared with the LB culture medium, so the cell density has been increased from 7.15 to 16.64 g/L and the titer of nanobody that produced is approximately sevenfold higher, from 12.23 to 78.43 μg/mL intracellularly and from 0 to 9.23 μg/mL extracellularly.

8.1 | Discussion

Temperature is a crucial factor that affects cell growth and protein production [14]. This factor also affects the substrate consumption rate, the quality of the product, and process duration. Since the culture medium temperature increases the rate of protein synthesis, it may not provide enough time for the correct protein folding [4, 15]. Many attempts have been made to improve folding and solubility during the expression. One of the most commonly used strategies is changing bacterial growth conditions [7]. Although the temperature drop to less than 37°C in *E. coli* causes a reduction in specific growth rates [16]. We investigated the effect of various temperatures on the yield of extracellular production of nanobody and cell biomass. Generally, low temperature leads to slower cell growth and protein expression. Decreased cell growth resulted in reduced consumption of carbon source and increased dissolved oxygen, which results in decreased acetate [16, 17]. SDS-PAGE analysis (Figure 6) revealed that glycine in autoinduction medium could effectively promote the release of recombinant nanobody, possibly due to its impact on fatty acids leading to increased cell membrane fluidity and permeability [1, 6, 18]. The expression of recombinant protein in the form of soluble and biologically active are advantages in comparison to the inclusion body (insoluble form) as the various steps, including refolding, could be avoided in downstream processing. In this study, the conventional induction (IPTG inducer) which produced insoluble recombinant protein in *E. coli* is compared with the autoinduction strategy for soluble expression of nanobody. Based on previous reports, the maximum amount of extracellular production occurred at the lower concentration of glucose and at the higher concentration of glycerol [7, 11, 19]. The high concentration of glucose in the medium causes catabolic repression and also the production of byproducts such as acetate [20]. Moreover, this causes the increase of acidity of the medium and reduction of target

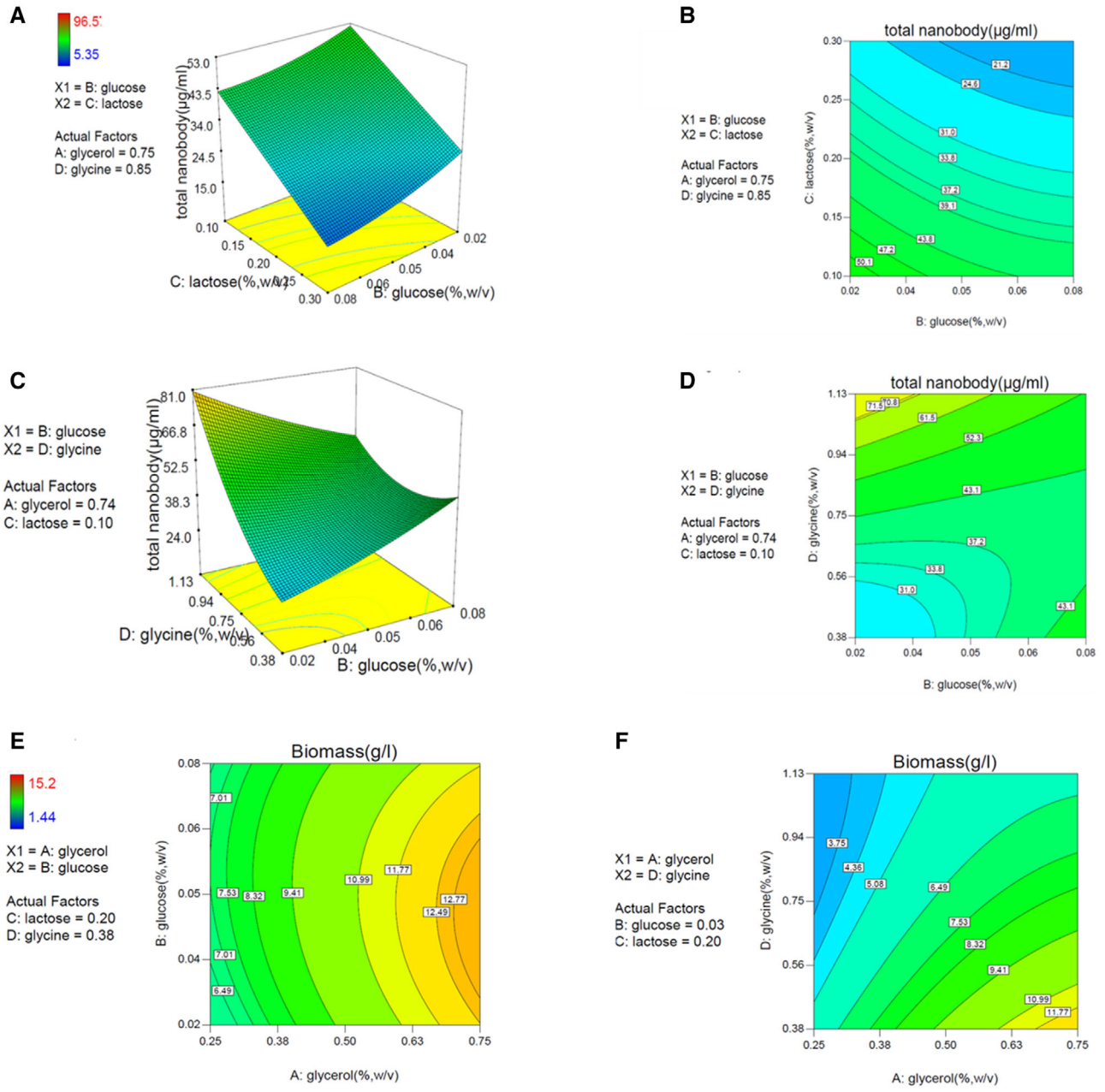

FIGURE 5 The contours and 3D plots for production of total nanobody including intracellular and extracellular and biomass production: (A) the effect of lactose and glucose, (B) the effect of lactose and glucose, (C) the effect of glycine and glucose, (D) the effect of glycine and glucose, (E) the effect of glucose and glycerol, and (F) the effect of glycine and glycerol (other carbon sources were kept constant for each experiment)

protein production [2]. In contrast to the results obtained for glucose, glycerol has a positive impact on target protein production. Also, the optimal response has occurred in the minimum amount of lactose in medium, which can be due to the loss of bacterial recombinant plasmid and bacterial death at high concentrations of lactose. The amount of extracellular production of nanobody was demonstrated by examining lactose and glucose (Figure 3C). According to the results (Figure 3), the optimal was obtained at low concentrations of both glucose and lactose. Excessive lactose in the culture medium increases the misfolding of the protein [19, 21]. The effects of glycine along with glucose, glycerol, and lactose on the extracellular production of nanobody are shown in Figure 3D and E. In general, for optimal concentration of glycerol, glucose, lactose and the lowest concentration of glycine, the highest amount of extracellular nanobody was produced. Glycine affects the cell wall of the bacterium and its direct effect on the peptidoglycan layer and increases the permeability of the cell wall. So, the protein is more easily secreted into the culture medium [6, 18, 22, 23]. Glycine plays two major roles in the increasement of nanobody production: (1) increase the expression of nanobody as described

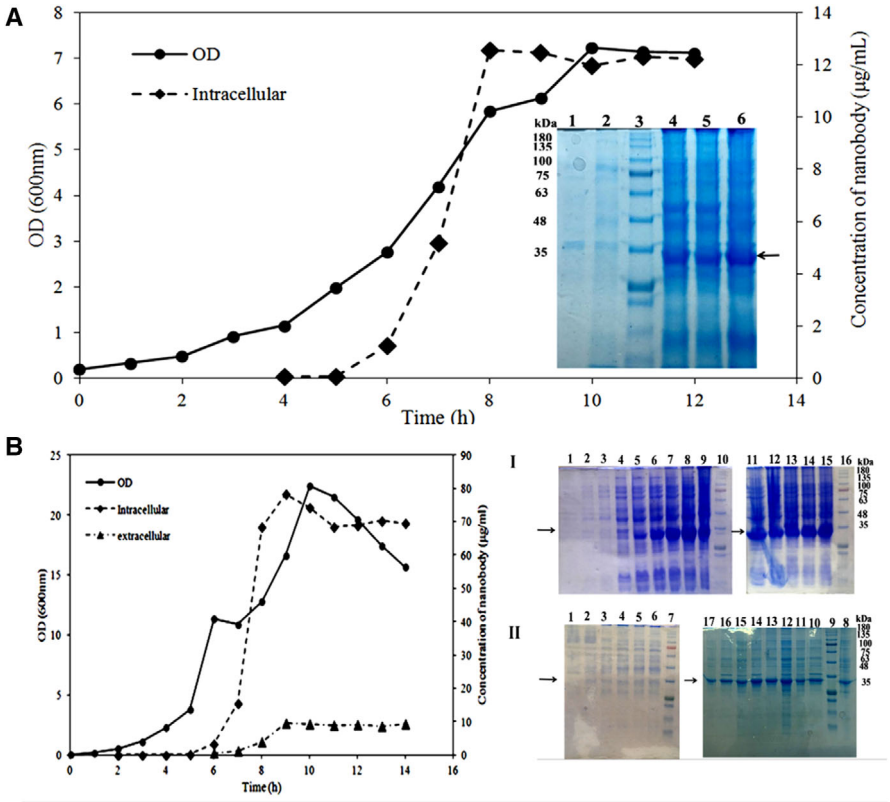

FIGURE 6 (A) The growth curve and nanobody production in LB medium and the presence of IPTG as inducer, SDS-PAGE analysis intracellular release of nanobody, (1) before induction, (2) induction time, (3) marker, (4–6) 2, 3, and 4 h after induction, respectively, (B) the growth curve and intracellular and extracellular production of nanobody in autoinduction medium in presence of lactose as inducer, PAGE analysis of (I) intracellular production, and (II) extracellular production of nanobody during cell culture, the number (1 to 17) shows the time (h) of culture. Arrows shows the target nanobody in the culture medium. *Lanes 9, 7, 10, and 16 are MW markers

before and, (2) it enhances the permeability of bacterial cell wall [5, 24]. Lower concentrations of glucose and lactose give higher production of total nanobody as reported by other researchers [6, 25]. In conclusion, this study reveals that the use of autoinduction conditions for protein expression under optimum conditions has a profound effect on the production of bispecific anti-MUC1 nanobody. Glycerol, glucose, lactose, and glycine were selected as the pivotal factors for the optimization of experimental conditions, all of which have impact on the acidity of culture media, process duration, cell density, as well as on the nanobody production. In addition, our findings revealed that temperature and time are two essential items in the production of nanobodies. Furthermore, the production of nanobody was investigated for both LB and optimized autoinduction media in the fermenter, and the results were compared. Our results have demonstrated that the optimized autoinduction medium is suitable for large-scale production of bispecific anti-MUC1 nanobody, as the amount of produced nanobody was increased sevenfold in a batch fermenter. Therefore, the results obtained in this study could be technically and economically interesting for pharmaceutical companies which produce recombinant proteins, specifically antibody fragments.

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CONFLICT of INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This research does not contain any studies with human participants or animals performed by any of the authors.

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