Original Article

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Evaluating the Effects of Environmental and Culture Conditions on Anti-HER2 scFv Expression and Solubility in *E. coli*

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ABSTRACT

Background & Objective: Breast cancer is an international concern due to its high prevalence worldwide. Human epidermal growth factor receptor 2 (HER2)-positive breast cancers were observed in 15-20% of breast cancers, with higher aggressiveness and poor prognosis. The single-chain fragment variable (scFv) structure includes variable regions of antibody light and heavy chains. Despite the promising potential of anti-HER2 scFv for non-invasive detection of HER2 status, its low expression and solubility remain significant challenges. Here, we intended to optimize the anti-HER2 scFv expression and solubility in BL21-pET-22 (anti-HER2 scFv).

Materials & Methods: The effects of ethanol stress, aeration, post-induction temperatures, induction-starting times, culture mediums, and $MgCl_2$ addition were examined.

Results: We observed that ethanol stress (1% v/v) increased protein expression and decreased protein solubility at 37 °C. However, it enhanced anti-HER2 scFv solubility at lower post-induction temperature (22 °C). Induction at OD_{600}=1 elevated anti-HER2 scFv expression, along with a substantial protein solubility enhancement at OD_{600}=0.6-0.8. In the LB medium, speeding up the shakers to 250 RPM led to a non-significant enhancement of anti-HER2 expression and a significant solubility improvement. The best medium for the optimum anti-HER2 scFv expression and solubility was the TB medium. $MgCl_2$ could not increase anti-HER2 expression or solubility.

Conclusion: The highest anti-HER2 scFv expression level was obtained when BL21-pET-22 (anti-HER2 scFv) was cultured in TB medium, induced at OD_{600}=2 and 37 °C, and incubated at 250 RPM. The highest solubility was also observed in the TB medium, induced at OD_{600}=0.6-0.8 and 37 °C, and incubated at 250 RPM.

Keywords: Breast Neoplasms, Antibody Fragments, HER2 (Receptor, ErbB-2), Protein expression, Solubility

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Introduction

Breast cancer accounts for the highest prevalence and mortality rate of cancers among women worldwide (1). The molecular pathogenesis of this malignancy is based on three main receptors, including estrogen (ERα), progesterone (PR), and epidermal growth factor 2 (ERBB2, HER2, or HER2/neu) receptors. HER2 is a transmembrane tyrosine kinase receptor. HER2 overexpression can be found in about 15-20% of breast cancers, resulting in poor prognosis, more angiogenesis, tumor aggressiveness, and higher prevalence of systemic and brain metastasis. HER2+ breast cancers can be treated by targeted therapy approaches such as anti-HER2 monoclonal antibodies (Trastuzumab, TD-M1, Pertuzumab), and small-molecule tyrosine kinase inhibitors (Lapatinib) (2-4).

Single-chain fragment variable (scFv) is a class of antibodies that consists of variable regions of light and heavy immunoglobulin chains that are connected by a

linking peptide. Regarding their structure and smaller size, they have higher diffusion abilities to tumor cells, specificity, affinity, and drug clearance compared to intact antibodies. They are producible in *E. coli* expression system (5, 6). *E. coli* bacterial host is inexpensive, fast, easy to use, and capable of producing high-levels of protein. Despite advantages, limiting factors, including protein insolubility and formation of inclusion bodies, low expression or purification yields, and protein inactivation make the process challenging (7, 8). Therefore, different strategies are used to eliminate these limitations of protein expression and solubility.

Ethanol is an amphipathic molecule that changes the bacterial membrane fluidity and may contribute to increased DNA synthesis and protein expression. It can also mimic heat-shock response in bacterial cells, induce hypoxia, and reduce aerobic metabolism. It contributes to slower biosynthesis of macromolecules, less misfolded protein expression, and enhances protein solubility. However, higher concentrations show anti-bacterial effect which is not desirable (9, 10).

Higher shaking speeds and the flask volume to liquid volume ratio cause higher aeration and oxygen transfer, and may enhance bacterial growth. However, some factors such as the production of toxic metabolites at higher growth rates can limit the effects of shaking speed on protein expression and solubility (11, 12).

The induction-starting time also influences protein expression. A metabolic burden can be imposed on the host strain associated with protein overexpression in early induction. Some studies demonstrated that due to higher cell densities, induction at high OD_{600} values results in increased yield of protein production. However, stressful conditions such as insufficient nutrients after the exponential phase may increase protease levels and consequently decrease protein expression. Several studies reported the positive effect of induction at lower OD_{600} in protein expression and solubility (13, 14).

It was found that culture medium composition can affect protein, plasmid, and cell growth yield. In this research, Luria-Bertani (LB), terrific broth (TB), and super broth (SB) mediums were evaluated. It was reported that TB medium can prolong the exponential phase of *E. coli* and increase plasmid yield, which improves bacterial growth and recombinant protein production (15-18).

Addition of metal ions, especially Mg_{2+} to culture medium, may enhance the solubility of some proteins based on metal-ion-dependent enzymatic activity that may result in the correct disulfide bond formation in E. coli. There are controversial findings about Mg_{2+} addition on protein expression and solubility (11, 17).

Regarding the poor prognosis of HER2 overexpression in breast cancer and the promising potential of radiolabeled anti-HER2 scFv in non-invasive detection of HER2 status in clinic (19), overcoming the challenges of its bacterial expression including a low level of expression and solubility is highly valuable. In our current study, we aimed to enhance the soluble expression of recombinant human anti-HER2 scFv in *E. coli* BL21 (DE3). Accordingly, the effectiveness of different approaches including various culture and induction conditions, and the addition of ethanol and Mg_{2+} to the culture medium were studied.

Materials and Methods

The bacterial strain BL21 (DE3) and the protein expression vector pET-22b (+) were generously gifted from Dr. Nematollahi and Dr. Behdani, respectively (Pasteur Institute of Iran). Ampicillin solution (100 mg/mL) was prepared from the sterile ampicillin powder. It was then added to the culture medium to obtain a 100 µg/mL concentration in the starter. All of the chemicals used in this study were purchased from standard resources including Sigma® and Merck®, otherwise they were mentioned in the text.

Culture medium preparation

LB is the common bacterial culture medium, while TB culture medium is supplemented with K_2HPO_4, KH_2PO_4, and glycerol as phosphate and carbon sources (15-18). To prepare different culture medium, the components of LB broth (yeast extract 0.5 %w/v (Quelab, Canada), tryptone 1% w/v (Quelab, Canada), NaCl 1% w/v) ,SB broth (yeast extract 2% w/v, tryptone 3.2% w/v, NaCl 0.5% w/v) ,and TB broth (yeast extract 2.4% w/v, tryptone 1.2 % w/v, glycerol 0.4% v/v, K_2HPO_4 72 mM, KH_2PO_4 17 mM) were dissolved in distilled/deionized water. Next, the pH of each medium was adjusted to 6.8-7.2 and then sterilized by autoclaving.

Anti-HER2 scFv protein expression and fractionation

BL21 (DE3) containing pET-22 (anti-HER2 scFv) was inoculated on an agar plate containing ampicillin (100 µg/mL) and the grown bacteria were used for an overnight pre-culture in broth medium containing ampicillin. The next day, pre-culture was added to the fresh broth medium supplemented with ampicillin to provide 30 mL bacterial culture at OD_{600}=0.1 as the starter culture. The starter culture was incubated in shaker incubator at 37 °C until reaching the OD_{600} to 0.6-0.8. Thereafter, it was induced by 0.25 mM IPTG (Sinaclon, Iran, biological grade, Cat No: CL5811). The bacterial cell harvesting was performed through a centrifugation step (10,000×g for 5 min) 24 h after induction. Then, the isolated bacterial pellet was

resuspended in 5 mL of lysis buffer (NaH_2PO_4, NaCl, imidazole, pH=8), mixed with lysozyme (Sigma, 1 mg/mL) and incubated on ice for 30 min. The cell lysate was sonicated for 10 min [300 W, 7 s working and 7 s resting, (Topsonics, Iran)] and then centrifuged (13000×g for 25 min) to isolate the soluble and insoluble protein fractions.

Optimization of protein expression and solubility

- Ethanol stress at different post-induction temperature

Ethanol 99% addition to the culture medium containing BL21-pET-22 (anti-HER2 scFv) was conducted at OD_{600}=0.1 to obtain final ethanol concentrations of 1, 2, 3% (v/v) (9, 20). Then, the starter cultures were prepared for each ethanol concentration and flasks were shaken in the incubator at 180 RPM at 37 °C until IPTG induction at OD_{600}=0.6-0.8. Next, the flasks were divided into two post-induction temperature groups incubating at 37 or 22 °C.

- Agitation rate and time of induction in LB medium

Six flasks containing diluted culture at OD_{600}=0.1 were prepared and divided into two groups: three flasks were incubated in a shaker incubator at 180 RPM and 37 °C, and others were placed into shaker incubator at 250 RPM and 37 °C. In each group, one sample was induced at OD_{600}=0.6-0.8, one at OD_{600}=1, and the latter at OD_{600}=2. Then, they were incubated in the same condition as before induction.

- Different culture medium

The overnight pre-cultures were prepared using different culture medium (LB, TB, and SB medium), and then they were diluted with the related medium to obtain an OD_{600}= 0.1. Then, the duration of incubation and shaking rate of the incubator were set according to the obtained results in LB medium. Four flasks for each culture medium were prepared. Three of them were incubated in the shaker incubating at 250 RPM and 37°C and were induced at OD_{600}=0.6-0.8, 1, and 2, while the other flask was incubated in shaker incubator at 180 RPM and 37 °C and was induced at OD_{600}=1.

- $MgCl_2$ addition to the culture medium

Sterilized $MgCl_2$ (0.1 M, Merck, Cat No: 7786-30-3) was added to the culture medium (LB, SB, and TB) at OD_{600}=0.1 at final concentrations of 10 and 30 mM. The culture flasks were incubated in a shaker incubator at 250 RPM and 37 °C, and were induced at OD_{600}=2.

Determination of anti-HER2 scFv concentration

The anti-HER2 scFv/total protein ratio was defined on 12.5% stained SDS-PAGE gel using Gel Analyzer software (version 19.1). Total protein concentration was calculated by BCA assay kit (Parstous®, Iran) using bovine serum albumin (BSA) as the standard. To calculate the concentrations of anti-HER2 scFv, as well as its soluble and insoluble fractions, the ratio of anti-HER2 scFv/total protein was multiplied by total protein concentration. Solubility was defined as the ratio of the mean concentration of soluble anti-HER2 scFv protein to the mean total protein concentration. Our data were analyzed statistically by Graph Pad Prism 8.0 software for Windows (Graph Pad Prism, San Diego, California, USA). Our findings were reported as mean ± standard deviation of two independent experiments. The data statistical analysis was conducted using one-way ANOVA with Tukey's post hoc test. The $p<0.05$ was considered to be statistically significant.

Results

The effect of ethanol stress on anti-HER2 scFv expression and solubility

Based on the findings of our previous studies, the molecular weight of the expressed anti-HER2 scFv protein was 28 kDa (15, 16). Protein expression was significantly increased by ethanol addition (1% v/v), when BL21-pET-22 (anti-HER2 scFv) was induced at 37 °C in comparison with ethanol-free medium (Table 1a, Figure 1a, Suppl. Figure 1a). However, further increase in ethanol concentration resulted in decreased anti-HER2 scFv expression, which was observed in both post-induction temperatures (37 and 22 °C) (Figure 1a, 1b, Suppl. Figure 1a and b). The concentration of soluble anti-HER2 scFv demonstrated a decreasing trend in response to increasing ethanol concentration (0-3% v/v) when recombinant *E. coli* was induced at 37 °C (Figure 1c, Suppl. Figure 1c and d). However, the soluble anti-HER2 scFv concentration, which was induced in the presence of ethanol (1% v/v) at 22 °C was significantly higher in comparison with that of induced at 37 °C (Table 1b, Figure 1c, Suppl. Figure 1c and d).

The effect of agitation rate, induction-starting time, and different culture mediums on anti-HER2 scFv expression

In LB medium, the recombinant BL21-pET-22 (anti-HER2 scFv) cells were incubated at 37 °C, and, IPTG-induction was performed at different OD_{600} = 0.6-0.8, 1, and 2. Under both aeration conditions, the highest protein expression was obtained at OD_{600}=1 insignificantly ($p>0.05$) (Figure 2a). Furthermore, increasing the oxygen supply by speeding up the shakers enhanced protein expression at 250 RPM compared to 180 RPM, which was statistically insignificant ($p>0.05$) (Figure 2a, Suppl. Figure 2a, b).

To evaluate SB and TB mediums, the experiments were conducted at 250 RPM. Accordingly, three flasks containing BL21-pET-22 (anti-HER2 scFv) were incubated at 37 °C, shaking at 250 RPM, and induced at OD_{600}=0.6-0.8, 1, and 2. In both SB and TB mediums, the highest anti-HER2 scFv expression level was observed in shaker incubator at 37 °C, 250 RPM, and when induced at OD_{600}= 2 (Figure 2b). The level of anti-HER2 scFv expressed under this condition was significantly higher compared to the LB medium. Culturing BL21-pET-22 (anti-HER2 scFv) in TB and SB mediums at 37 °C, 250 RPM, induced at OD_{600}= 2, resulted in 4.3 and 2.35 times increase in protein expression (Table 1a) in comparison with the control group (LB medium induced at OD_{600}= 0.6-0.8 shaking at 250 RPM), respectively. Furthermore, culturing in TB compared to LB medium led to significantly enhanced anti-HER2 scFv expression in all of the examined induction-starting times (OD600=0.6-0.8, 1 and 2) (Figure 2b and c).

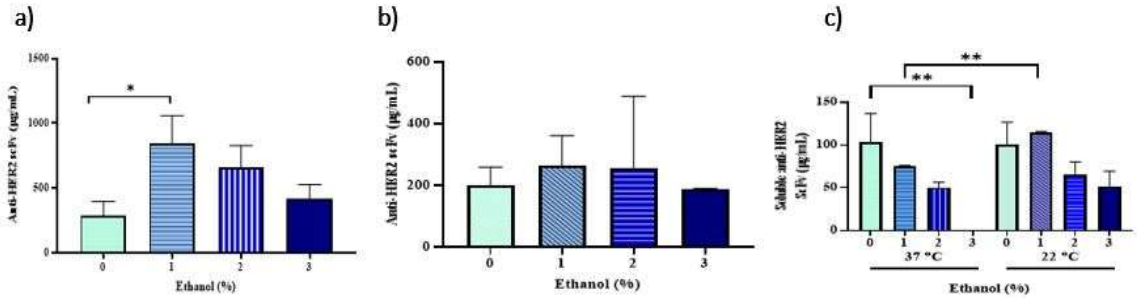


Figure 1. Effect of ethanol stress on anti-HER2 scFv expression and solubility in LB medium. Anti-HER2 scFv expression by BL21-pET-22 (anti-HER2 scFv) induced by IPTG (0.25 mM) at a) 37, and b) 22 °C in the presence of ethanol (1-3% v/v), and c) soluble anti-HER2 scFv expressed by BL21-pET-22 (anti-HER2 scFv) and induced, at 37, as well as 22 °C in the presence of ethanol (1-3% v/v). Results are reported in the mean ± SD (n=2) format. *, **, * indicate p<0.05, p<0.01 and p<0.001, respectively.**

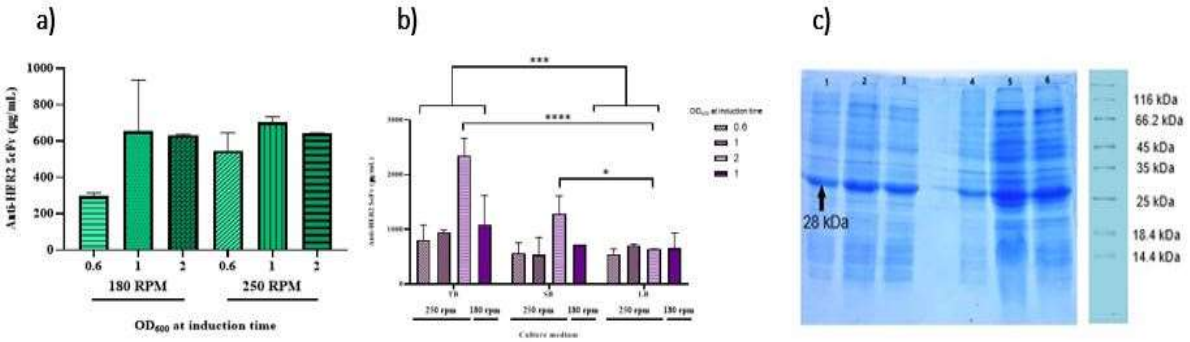


Figure 2. Effect of agitation rate, induction-starting time and culture medium on anti-HER2 scFv expression. Anti-HER2 scFv expressed by BL21-pET-22 (anti-HER2 scFv) and induced by IPTG (0.25 mM) at OD_{600}= 0.6-0.8, 1, and 2 in a) LB medium shaking at 180 and 250 RPM, and b) in LB, TB, and SB mediums shaking at 250 RPM as well as at OD_{600}= 1 in 180 RPM. c) SDS-PAGE analysis of anti-HER2 scFv expressed by BL21-pET-22 (anti-HER2 scFv) and induced at OD_{600}=2, 37 °C, shaking at 250 RPM 24 h after IPTG-induction in the 1) SB, 2) TB, 3) LB culture mediums, and 2 h post-induction in 4) SB, 5) TB, 6) LB culture mediums. Results are reported in the mean ± SD (n=2) format. *, *, and **** represent for p<0.05, p<0.001 and p<0.0001, respectively.**

The effect of agitation rate, induction-starting time, and different culture mediums on anti-HER2 scFv solubility

In LB medium, anti-HER2 scFv solubility was influenced by induction-starting time and agitation rate. The highest soluble anti-HER2 scFv concentration was achieved when the induction was performed at OD_{600}=0.6-0.8. Although no significant difference was observed between soluble anti-HER2 scFv concentrations induced at different starting times in TB (Figure 3b) and SB (Figure 3c) mediums, the highest solubility was observed in the earliest induction-starting time (OD_{600}=0.6-0.8) in all the examined mediums.

In LB medium, soluble anti-HER2 scFv was significantly increased, when oxygen supply was increased from 180 to 250 RPM and IPTG induction was performed at OD_{600}=0.6-0.8, (Figure 3a, Suppl. Figure 2c). Statistically insignificant (p>0.05) enhancement of soluble anti-HER2 scFv concentration by increasing agitation rate was also observed in TB (Figure 3b) and SB mediums (Figure 3c).

Culturing BL21-pET-22 (anti-HER2 scFv) in the TB culture medium, with a shaking rate of 250 RPM, and IPTG induction at OD_{600}=0.6-0.8 resulted in the highest solubility that was 11.5 times higher than the control group (Culturing in LB, with a shaking rate of 250 RPM and IPTG-induction at OD_{600}=0.6-0.8) (Table 1b, Figure 3d).

The effect of adding $MgCl_2$ to the culture medium on anti-HER2 scFv expression and solubility

As demonstrated in Figure 4a, $MgCl_2$ addition to LB and SB medium did not affect the expression of anti-HER2 scFv protein. Conversely, anti-HER2 scFv expression in TB medium was significantly reduced by 30 mM $MgCl_2$ (Figure 4a, Suppl. Figure 3a-c). Although the soluble part of anti-HER2 scFv was not considerably influenced by $MgCl_2$ addition in any mediums (Figure 4b, Suppl. Figure 4a, 4b), the anti-HER2 scFv solubility was increased drastically in TB medium in the presence of 30 mM $MgCl_2$ which may be due to its lower expression under this condition.

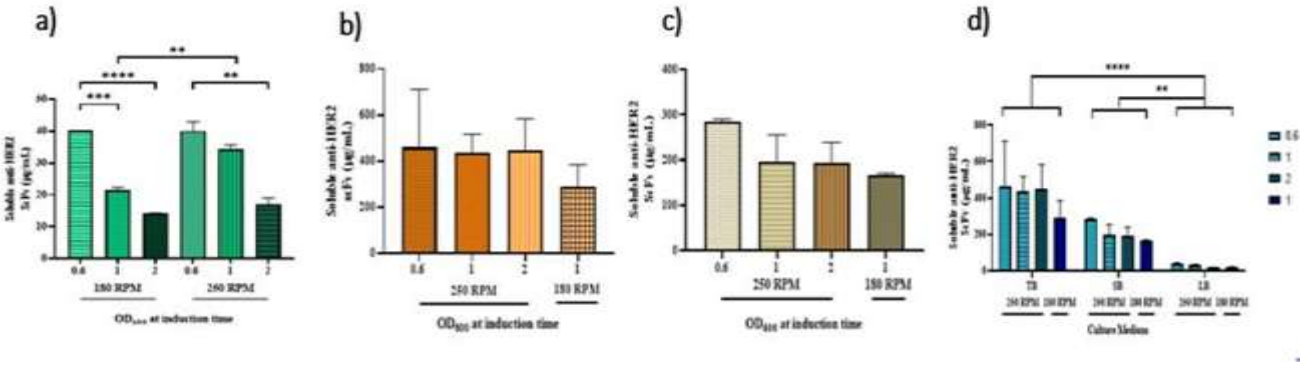


Figure 3. Effect of agitation rate, induction-starting time, and different culture mediums on anti-HER2 scFv solubility. Soluble anti-HER2 scFv expression by BL21-pET-22 (anti-HER2 scFv), induced by IPTG (0.25 mM) at OD_{600}=0.6-0.8, 1, and 2 and shaking at 180 and 250 RPM in a) LB, b) TB, and c) SB mediums. d) Comparison of soluble anti-HER2 scFv expression by BL21-pET-22 (anti-HER2 scFv) in TB, SB, and LB medium induced at OD_{600}= 0.6-0.8, 1, and 2 in 250 RPM, and at OD_{600}= 1 in 180 RPM. Results are reported in the mean ± SD (n=2) format. **, *, **** indicate p<0.01, p<0.001, p<0.0001, respectively.**

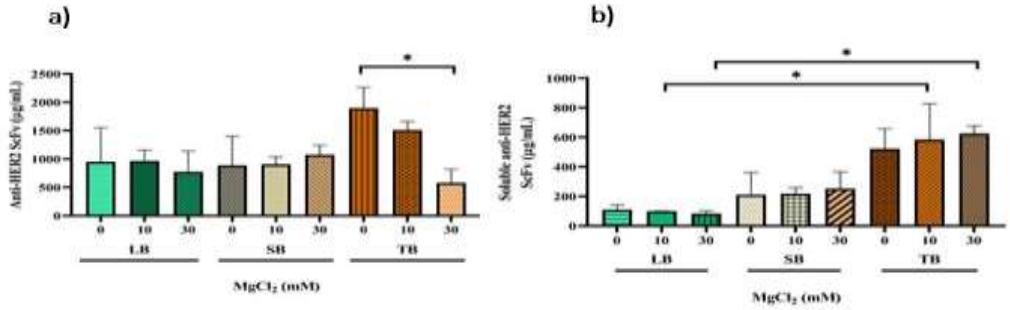


Figure 4. Effect of $MgCl_2$ addition (0, 10, and 30 mM) on anti-HER2 scFv expression and solubility. a) Anti-HER2 scFv expression by BL21-pET-22 (anti-HER2 scFv) in LB, SB, and TB mediums induced by IPTG (0.25 mM) at OD_{600}= 2, 250 RPM, in the presence of $MgCl_2$. b) Soluble anti-HER2 scFv expression by BL21-pET-22 (anti-HER2 scFv) in LB, SB, and TB mediums induced at OD_{600}= 2, shaking at 250 RPM, in the presence of $MgCl_2$. Results are reported in the mean ± SD (n=2) format. * represents for p<0.05.

Table 1. Significant effects of expression conditions on a) anti-HER2 scFv expression and its b) solubility

a)

Expression Condition	Anti HER2 scFv (µg/mL)	Fold Increase
LB medium, IPTG-induction (0.25 mM) at 37 °C- adding ethanol 1%	848.167	× 3

LB medium, IPTG-induction (0.25 mM) at 37 °C - ethanol free (Control)	282.378	
SB medium, IPTG-induction (0.25 mM) at OD_{600}=2, 37 °C and 250 RPM	1287.81	× 2.35
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM (Control)	547.763	
TB medium, IPTG-induction (0.25 mM) at OD_{600}=2, 37 °C and 250 RPM	2350.32	× 4.30
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM (Control)	547.763	

b)

Expression Condition	**Soluble anti HER2 scFv (µg/mL)**	**Fold Increase**
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 22 °C and 180 RPM- adding ethanol 1%	114.527	× 1.54
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 180 RPM- adding ethanol 1% (Control)	74.5925	
SB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM	285.057	× 7.11
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM (Control)	40.099	
TB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM	461.213	× 11.5
LB medium, IPTG-induction (0.25 mM) at OD_{600}=0.6-0.8, 37 °C and 250 RPM (Control)	40.099	

Discussion

The treatment outcomes have improved in HER2-positive breast cancer patients, especially early diagnosed and non-metastatic cancers by applying anti-HER2 monoclonal antibodies, small-molecule tyrosine kinase inhibitors, and antibody-drug conjugates (21, 22). Recently, we expressed biologically active anti-HER2 scFv in the *E. coli* BL21 (DE3) and observed the highest protein expression yield in BL21-pET-22 (anti-HER2 scFv) 24 h post-induction with 0.25 mM IPTG at 37 °C (23). Herein, the effects of ethanol stress, culture medium, induction-starting times, agitation rates, and $MgCl_2$ addition on the anti-HER2 scFv expression and solubility were evaluated.

Ethanol stress affects bacterial cell membrane's fluidity, transportations, and lipid and protein composition, increases DNA synthesis, and thus causes overexpression of the inducible proteins. The influence of ethanol's oxidative stress on main bacterial metabolic pathways also affects protein expression (20, 24). Furthermore, different studies demonstrated that ethanol stress increases heat shock protein synthesis, mimics their response, especially at low temperatures, and consequently improves protein solubility and stability (9, 25). In the current research, anti-HER2 scFv protein expression was increased significantly in the presence of ethanol stress (1% v/v) at 37 °C compared to the control group (ethanol-free) (Table 1a, Figure 1a). However, due to increased inclusion body formation as a result of ethanol stress, anti-HER2 scFv solubility was significantly decreased (Figure 1c). Mohammadinezhad *et al.* also observed that ethanol addition increased total infectious hematopoietic

necrosis virus (IHNV) nucleoprotein expression in both soluble and insoluble fractions, but could not enhance its overall solubility (25). Confirming our findings, Rani *et al.* revealed that ethanol addition (3% v/v) did not affect the soluble expression of human glycerol kinase in *E. coli* (26).

Herein, the anti-HER2 scFv protein expression was decreased when the culture medium was supplemented with higher concentrations of ethanol in response to its antibacterial effects (2 and 3 % v/v) (Figure 1a).

Post-induction temperature affects recombinant protein expression and solubility. Higher temperatures increase bacterial growth rate and protein expression. They also increase the possibility of plasmid loss and expression destruction. Hence, lowering the induction temperature leads to higher protein solubility and decreased protein aggregation (27, 28). Different studies indicated that lowering the induction temperature slows down the bacterial cell processes, weakens the temperature-dependent hydrophobic bonds, and consequently results in increased protein solubility and the soluble and active protein fraction yield in many proteins (29-32). In this research, the expression of total and soluble anti-HER2 scFv induced at 22 °C was increased under ethanol stress (1% v/v) slightly (Figure 1b, and c). We also observed that the soluble fraction of anti-HER2 scFv induced at 22 °C under ethanol stress (1% v/v) was significantly higher in comparison with induction at 37 °C (Figure 1c).

In most cases, IPTG induction at the early-mid log phase leads to the highest protein expression, while induction at higher cell densities (late-log phase and stationary phase) affects protein expression negatively due to less available nutrients, dissolved oxygen, and higher levels of acetate and carbon dioxide (30).

Herein, in LB medium, shaking at 180 RPM and 250 RPM, total anti-HER2 scFv expression was increased by delaying the induction-starting time from OD_{600}= 0.6-0.8 to OD_{600}= 1, and 2 (Figure 2a). Totally, in both agitation rates, the inclusion body formation was increased and the protein solubility was decreased significantly when induction time was delayed from 0.6-0.8 to OD_{600}= 1 and 2 (Figure 3a). The highest protein expression in the LB medium occurred when the *E. coli* BL21 was induced at OD_{600}= 1 (Figure 2a, b), while the highest protein solubility occurred when the *E. Coli* BL21 was induced at OD_{600}= 0.6-0.8 (Figure 3a). Confirming our observations, Van Noi *et al.* showed that IPTG induction at the mid-exponential phase at OD_{600}= 0.6 resulted in high levels of protein solubility followed by a descending pattern by induction at the stationary phase (33). In addition, Malik *et al* also indicated that cHSPA6 expression was approximately stable while IPTG induction during the exponential phase, while it was reduced at the stationary phase. They observed the highest protein solubility while inducing at mid-exponential phase (34).

There are controversial results regarding the effect of increasing oxygen supply on protein expression and solubility. In the current study, speeding up the shakers led to non-significant and significant enhancement of anti-HER2 expression and solubility in LB medium, respectively. Confirming our results, Collins *et al.* stated that agitation rate enhancement to 200-250 RPM and to 1:10 culture to flask volume ratio increased the silk-elastin-like proteins (SELP) protein expression in BL21. Conversely, Boshtam *et al.* reported that lowering the agitation rate from 220-250 RPM to 180 RPM resulted in the highest protein yield in *E. coli* BL21 due to the slower bacterial growth and proper protein folding (12, 35).

Cell growth and protein production are affected by culture medium composition based on its effects on bacterial physiology and stress during growth phases (36). Different studies have reported enhanced protein expression using rich culture medium such as TB and SB medium compared to LB medium, based on high nutrients, rich carbon sources, and a buffered environment (12, 18). In our study, the highest protein expression level was achieved while using TB medium, incubated at 250 RPM at 37 °C, and induced at OD_{600}=2 (Figure 2b). Furthermore, the highest solubility was also observed in the TB medium, incubated at 250 RPM at 37 °C, and induced at OD_{600}= 0.6-0.8 (Figure 3d).

Furthermore, SB medium also showed a higher level of anti-HER2 scFv expression compared to the LB medium (Figure 2b). Zamani *et al.* and Priyanka *et al.* observed that the highest cell concentration and recombinant protein production in BL21 (DE3) was achieved in TB medium (18, 37). Furthermore, Damough *et al.* observed higher levels of soluble TNFα expressed in TB medium in *E. coli*, compared with LB medium (38).

In this study, no expression enhancement was observed as a result of $MgCl_2$ addition to the culture mediums (Figure 4a). However, Luo *et al.* reported 5 times enhancement in protein expression by adding 10-30 mM $MgCl_2$ to the culture medium which affected RNA polymerase activity (17).

Moreover, there are other approaches including recombinant protein co-expression with chaperones, expression of affinity tag fusions, and various host systems that can be applied to improve the soluble protein expression. For instance, co-expression of ani-HER2 scFv and chaperones DnaK/DnaJ/GrpE at low temperatures improved protein folding and solubility (39, 40).

Altogether, we assumed that the effect of modifications on protein expression and solubility is a complex subject that requires optimization for each protein and culture condition. The necessity of optimizing various fermentation conditions (e.g., pH value, temperature, shear stress, and dissolved oxygen concentration) in bioreactors should not be overlooked. This may give rise to improved yield and efficiency of large-scale protein production (41). It should be taken into consideration that the methods that are expensive, time-consuming, or require complicated interventions cannot be applied on an industrial scale (42).

Conclusion

The optimum anti-HER2 scFv expression was observed when the *E. coli* BL21 host was cultured in TB medium, incubated at 250 RPM at 37°C, and induced at OD_{600}=2. The mean total protein expression in the optimized situation was 4.3 times higher than the control (LB, 250 RPM, 37 °C, induction at OD_{600}= 0.6-0.8). The highest anti-HER2 scFv solubility was achieved when the *E. coli* BL21 is cultured in TB medium, incubated at 250 RPM at 37 °C, and induced at OD_{600}= 0.6-0.8. The mean soluble protein expression reached 11.5 times higher than the control (LB, 250 RPM, 37 °C, induction at OD_{600}= 0.6-0.8). It is highly suggested to study other factors that may contribute to improved protein expression and solubility and predict the optimal expression conditions for small- and large-scale protein production using different statistical design approaches such as response surface methodology (RSM), in which the possible interactions between experimental variables are also evaluated (41).

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Conflict of Interest

The authors declare no conflict of interest.

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Ethics approval and consent to participate

No ethical experimentation (human/animal) was performed in this study. This project was found to be in accordance to the ethical principles for conducting medical research in Iran.
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Authors' contribution

T.M. performed the experiments. T.M. and E.M. wrote the main manuscript text. E.M. and A.S. supervised the project. All authors reviewed the manuscript.

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