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Original Research Article

Effects of Cultivation Conditions on the Expression Level of Recombinant scFv Antibody against EpEX in *Escherichia coli*

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HIGHLIGHTS

- The antiEpEX-scFv was successfully expressed in *E. coli* Rosetta™(DE3) strain.
- The highest concentration of protein was obtained with 0.5 mM IPTG at 30°C.
- The final yield of recombinant antiEpEX-scFv was approximately 403.29 ± 87.50 mg/L.

ABSTRACT

The EpCAM (epithelial cell adhesion molecule) is a cell surface antigen over expressed in many types of epithelial cell cancers including colon, stomach, pancreas, lung, ovarian, and breast. So, it can be an attractive target for active and passive immunotherapy of cancers. ScFv (single chain fragment variable) fragment is a class of engineered antibodies in which the genes coding for the heavy (V_H) and light chains (V_L) of an immunoglobulin have been linked with a short flexible peptide linker. Inexpensive media, rapid growth rates, and relatively minimal laboratory set up make *Escherichia coli* (*E. coli*) a suitable host for expression of a large variety of recombinant proteins. Here, we assessed the effect of cultivation conditions on the level of expressed scFv against extracellular domain of EpCAM (EpEX) in *E. coli*. pET22b-antiEpEX-scFv was transformed into prepared *E. coli* Rosetta™(DE3) competent cells. To evaluate the effect of cultivation conditions on protein expression level, three factors of incubation temperature (25, 30, 37°C), the IPTG (isopropyl-β-D-thiogalactoside) concentration (0.1, 0.25, 0.5, 1 mM), and induction duration (3, 5, 7, 18 h) were considered. SDS-PAGE and western blot analysis demonstrated an estimated 30 kDa-size protein band which was related to the recombinant scFv expressed in *E. coli* Rosetta™(DE3) strain. At optimal condition (5 h after induction with 0.5 mM IPTG at 25 °C), the final production yield of the antiEpEX-scFv was 403.29 ± 87.50 µg/mL. Our results provide a foundation for the development of scFv-based drugs' production as effective therapeutic agents in cancers with epithelial origin.

Introduction

The epithelial cell adhesion molecule (EpCAM) is a 40-kDa, transmembrane, 314-amino acid-long glycoprotein (Karabulut et al., 2014). In addition to cell migration, it is involved in cell adhesion, proliferation, and differentiation. However, its function is still largely unknown (Tas et al., 2014). Although EpCAM is overexpressed in various epithelial tumors such as colon, breast, rectum, biliary tract, stomach, prostate, pancreas, and hepatocellular, it is also expressed in the corresponding normal epithelia. However, in normal epithelia, the expression level of EpCAM is lower than

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what was found in tumor cells (Schmetzer et al., 2012). So, EpCAM can be potentially utilized as a suitable target for passive immunotherapy with monoclonal antibodies (mAb). Since the 1980s frequent studies were done to investigate the effectiveness of anti-EpCAM antibodies based targeted therapy. MAb17-1A, an anti-EpCAM monoclonal antibody with a low affinity was successfully used for colon and breast carcinoma therapy in Germany (Kirchner et al., 2002). Moreover, a bispecific scFv against EpCAM named CD3/17-1A showed an effective *in vitro* cytotoxicity to tumor cells (Mack et al., 1995). Finally, for the treatment of the patients with malignant ascites, European Union has approved catumaxomab which is a trifunctional monoclonal antibody against EpCAM/CD3 (Burges et al., 2007). Recent advances in recombinant antibody technology could facilitate designing and producing a wide variety of engineered antibody molecules including Fv fragments, Fab fragments, and scFv (single chain fragment variable) fragments in which the genes coding for V_H and V_L chains of an immunoglobulin molecule have been linked with a disulfide bond or a short flexible peptide linker. Better tumor penetration, lower retention times in nontarget tissues, more rapid blood clearance, and reduced immunogenicity are some of the several advantages of these minimized antibodies compared with the parental ones (Wang et al., 2015). Previously, scFv fragments were proved to be effective in treatment of the cancers in which the EpCAM receptor was overexpressed. Bispecific and fully humanized scFv fusion proteins against extracellular domain of EpCAM (EpEX) have been investigated in preclinical studies and clinical trials (Brischwein et al., 2006). Moreover, suitable *in vitro* antitumor activities were reported for several anti-EpCAM immunotoxins previously (Di Paolo et al., 2003).

Recently, the genes encoding several scFv fragments have been successfully cloned and expressed in bacteria, yeast, plant, and also mammalian cells. Inexpensive media, rapid growth rates, and relatively minimal laboratory set up make *E. coli* a suitable host for expression of a large variety of recombinant proteins (Wang et al., 2015, Agha Amiri et al,. 2017, Slouka et al., 2019). Moreover, using this favorable bacterial host, high antibody titers have been obtained (up to 2 g/L) (Chen et al., 2004).

Here, for the first time, a scFv from 4D5MOC-B humanized antiEpEX monoclonal antibody was expressed in *E. coli* Rosetta™(DE3) strain. The effect of temperature and IPTG concentration on the level of expressed antiEpEX-scFv was also evaluated.

Materials and Methods

Materials

Escherichia coli (DH5α) was used for plasmid preparation and *E. coli* Rosetta™(DE3) was used as a host for recombinant scFv expression (Pasteur institute of IRAN, Tehran, Iran). Luria–Bertani (LB) medium [0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 1% (w/v) NaCl, pH 7.0] supplemented with the antibiotic ampicillin (100 μg/mL) was used for growing the *E. coli* strains. Restriction endonucleases and T4 DNA ligase were purchased from Thermo Fisher Scientific (USA). Standard commercial sources have provided all used reagents and chemicals.

The antiEPEX -scFv expression

E. coli strain of Rosetta™(DE3) was transformed with previously developed pET22b vector harbouring the codon optimized antiEpEX-ScFv gene (pET22b-antiEpEX-scFv). For cultivation of host harboring pET22b-antiEpEX-scFv, a single colony was inoculated into 3 mL of Luria–Bertani (LB) broth containing 100 μg/mL ampicillin. After overnight shaking at 37 °C, the culture was transferred to LB medium containing 100 μg/mL ampicillin at a ratio of 1:10. Once the cell density (OD) reached 0.7–0.9, 1mM IPTG (Sinaclon, Iran) was added to the culture which was shaken for another 3, 5, 7, and 18 h under the same conditions.

Effects of cultivation conditions on antiEpEX-scFv expression

To evaluate the effect of culture condition on protein expression level, three factors of incubation temperature (25, 30, 37°C), the inducer concentration (0.1, 0.2, 0.5, 1 mM) and induction duration (3, 5, 7, 18 h) were considered. The cell pellets were harvested by centrifugation at 10000 rpm for 5 min.

SDS-PAGE and western blot analysis

The expression of recombinant protein was assessed by standard SDS-PAGE method. A c-terminal poly histidine tag was employed for detection of recombinant protein in designed construct. For western blotting, after separation by 12% SDS-PAGE, the bacterial lysates were transferred to PVDF membrane. 2% bovine serum albumin (BSA) in phosphate-buffered saline with 0.1% Tween 20 (PBST) was used to block the transferred membrane. 1/10000 dilution of anti-his tag polyclonal antibody was used as the primary antibody (Sigma, UK). After washing, membrane was incubated in a 1.5/10000 dilution of anti-mouse HRP conjugated immunoglobulin (Sigma, UK) as the secondary one. The blot was developed using 3, 3'-diaminobenzidine (DAB) substrate. Utilizing TL120 software (Nonlinear Inc, Durham, USA), the protein expression level was assessed by densitometry analysis of poly acrylamide gels.

Determination of protein concentration

The total protein concentrations were determined via the bicinchoninic acid (BCA) assay and BSA was used as

standard (Takara, Japan). For sample preparation, the total bacterial pellets were lysed in lysis buffer (100 mM NaH2PO4, 10 mM urea, pH= 8). The standard curve was generated based on concentrations of BSA standard samples. The concentration of the total protein was determined using the standard curve as a reference. Total protein samples were also electrophoresed on a 12% SDS-PAGE gel. The band intensity of recombinant protein was analyzed using TL120 software (Nonlinear Inc, Durham NC, USA). Based on estimated intensity, TL120 calculates the quantity of recombinant protein as a percentage of total protein. According to total protein concentrations obtained from BCA assay and percentage of recombinant protein obtained from TL120 analysis, the concentration of recombinant protein can be calculated.

Results and Discussion

Expression and detection of antiEPEX-scFv protein

The pET22b vector harboring the codon optimized antiEPEX-ScFv gene (pET22b-antiEPEX-scFv) was transformed into Rosetta™(DE3) competent cells. When recombinant proteins are expressed in *E. coli*, translation may be hampered because of shortage or lacking for one or more tRNAs. So, two strategies can be considered to improve heterologous protein expression in *E. coli*; codon optimization in target gene and utilization of engineered hosts which can supply the rare tRNAs during the expression (Kong and Guo, 2014). Previously published data has shown a significant promotion in protein level of eukaryotic genes expressed in *E. coli* after codon-optimization. For example, after codon optimization, higher amount of recombinant fGH was achieved in *E. coli* (Choi and Geletu, 2018). Overcoming this disparity, numerous bacterial strains such as Rosetta™(DE3) has been also developed. *E. coli* strain Rosetta™(DE3) which carries tRNA for rare codons including AUA, AGG, CUA, AGA, GGA, and CCC has been successfully employed by several studies. For example, when human β-defensin 2 (hBD2) gene was expressed in Rosetta™(DE3) strain, a nine-fold increase has been reported in its expression level by Peng et al. (2004). Introducing T7 polymerase encoding gene in *E. coli* strain Rosetta™(DE3) genome makes this host suitable for expression of genes cloned in pET vectors with T7 promoter. Here, the antiEPEX-scFv gene was expressed in pET22b (+) vector under the strong T7 promoter system in *E. coli* Rosetta™(DE3). Up to now, *E. coli* Rosetta™(DE3) was successfully utilized to improve expression of recombinant scFvs. As an example, the engineered scFv-mms13 fusion protein was successfully expressed in this expression host (Kong et al., 2015).

After cultivation at the 1 mM IPTG at 37°C, the bacterial cells were lysed and analyzed via SDS PAGE. Compared to the negative control, the protein band was detected at the theoretically expected molecular weight (30 kDa) (Fig. 1).

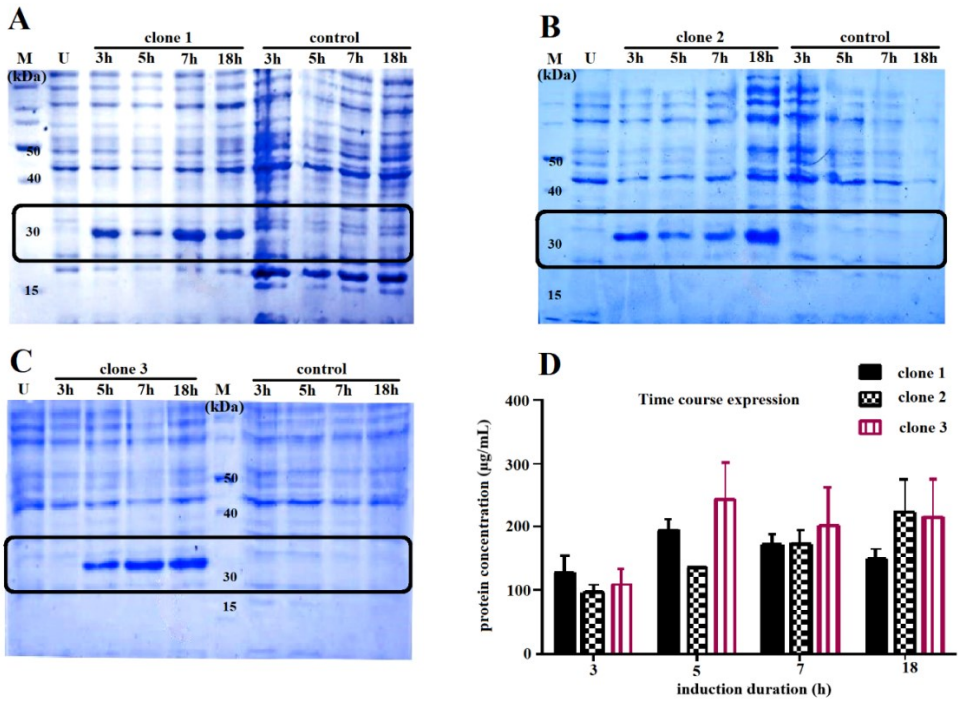

Figure 1. Expression analysis of the antiEPEX-scFv protein using SDS-PAGE. The expression level of target protein was examined in three clones. Proteins were separated on a 12 % SDS-PAGE gel and visualized by coomassie brilliant blue R250 staining. Total protein was extracted from *E. coli* Rosetta™(DE3) containing pET22b-antiEpEX-scFv plasmid before induction (U) and after induction with 1 mM IPTG for 3h, 5h, 7h and 18h respectively at 37 °C (M: protein marker). Control is cells transformed with pET22b.

The expression level of target protein was examined in three clones. By screening various incubation times (3, 5, 7, 18h), the highest protein expression level was obtained at 5 h after induction in clone 3 (Fig. 1). As shown in fig. 1 D, based on densitometry analysis and BCA assay, maximum level of expressed recombinant scFv was estimated up to 194.45 µg/mL (at 5 h after induction) 223.13 µg/mL (at 18 h after induction) and 243.09 µg/mL (at 5 h after induction) in three clones examined respectively. However, the expressed protein had a higher level (243.09 µg/mL) in clone 3 (Fig. 1 D). Using anti-his antibody, the western blotting analysis indicated that expressed antiEPEX-scFv was a his-tagged fusion protein (Fig. 2).

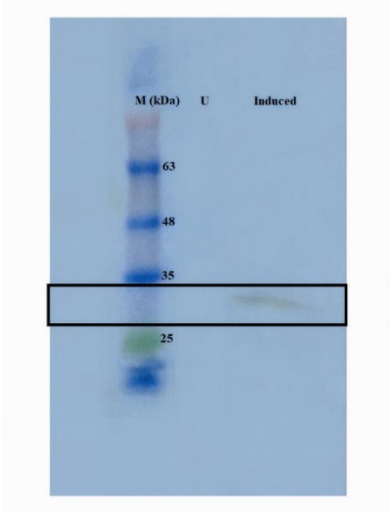

Figure 2. Western blotting analysis of the recombinant antiEPEX-scFv protein. Bacterial lysates of *E. coli* Rosetta™(DE3) before (U) and after induction (In) were treated with the anti His monoclonal antibodies (M: pre-stained protein marker). The antiEPEX-scFv protein (30-kDa) is denoted.

Effects of temperature and IPTG concentration on the expression level of antiEPEX-scFv

The concentration of IPTG needs to be optimized because on the one hand, it has a determining role in protein expression level, but on the other hand, it causes serious harm to cell growth (Rasouli kery bozorg and Hashemi, 2019). In this study, the effect of different IPTG concentrations (0.1, 0.25, 0.5 or 1 mM) on expression level of recombinant protein was examined (Fig. 3). Moreover, based on many reports demonstrated that the growth temperature had the most serious impact on improving protein production in the *E. coli* B strains (Rasouli kery bozorg and Hashemi, 2019), here, the effect of different temperatures (25, 30, 37°C) on anti EpEX-scFv expression level was also evaluated. Based on densitometry analysis as well as BCA assay, the highest level of expressed antiEPEX-scFv was achieved by 0.5 mM IPTG induction at 25°C for 5 h (Fig. 4). As shown in fig. 4, the productivity of antiEPEX-scFv protein was decreased using higher IPTG concentration (1mM) at this temperature. In accordance with our results, Zheng et al. reported a significant decrease in the expression level of acetolactate synthase in *E. coli* when the IPTG concentration was more than 0.6 mM (Zheng et al., 2015). Similarly, in two previously published studies, an improved expression of recombinant form of one major mango allergen (rMan i 1) as well as recombinant pneumococcalsurface adhesin A (rPsaA) in *E. coli* was obtained when a low concentration of IPTG was used for induction (Larentis et al., 2011, Tsai et al., 2017). The results obtained in our study also are in accordance with data reported by Lim *et al.* which demonstrated an inhibition in the expression of scFv against anti-exotoxin when more than 1 mM IPTG was used for induction (Lim et al., 2004). The IPTG toxic effect may be due to an induction of bacterial proteases which may degrade recombinant proteins. Proteases were also able to enhance metabolic load (Lim et al., 2004).

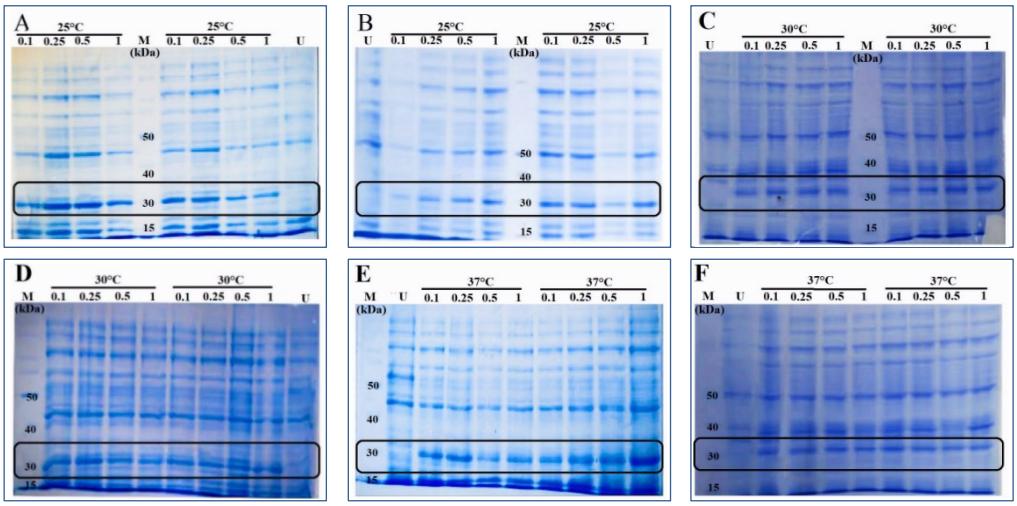

Figure 3. The effects of IPTG concentration (mM) and incubation temperature on antiEPEX-scFv protein expression in *E. coli* Rosetta™(DE3). After separation on a 12 % SDSPAGE gel, protein bands were visualized by coomassie brilliant blue R250 staining. Protein expression was induced with different concentrations of IPTG (0.1, 0.25, 0.5, and 1 mM) at A and B: 25 °C, C and D: 30 °C, and E and F: 37 °C for 5 h. Lane M: protein marker. Lane U: bacterial culture before induction. The position of antiEPEX-scFv was indicated.

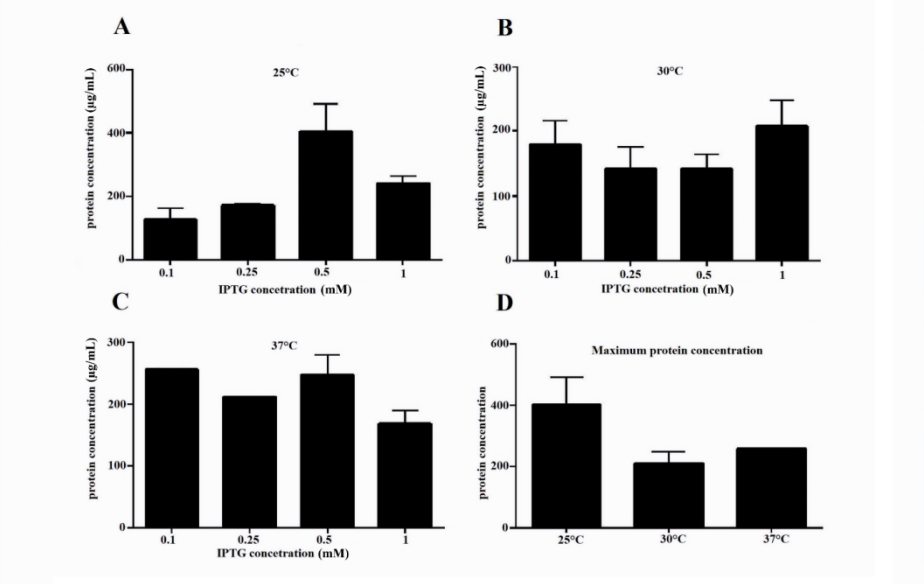

Figure 4. Comparison of the concentration of the expressed antiEPEX-scFv depending on different IPTG concentrations (mM) and incubation temperatures. Protein expression was induced with different IPTG concentrations (0.1, 0.25, 0.5, and 1 mM) at A: 25 °C, B: 30 °C, and C: 37 °C for 5 h. D: The maximum protein concentration obtained from A, B and C has been compared.

The final yield of the recombinant protein can also be affected by temperature via influencing on mRNA expression rate, protein folding, aggregation, and secretion (Gupta and Shukla, 2016). Here, the maximum level of recombinant protein was attained at a low temperature of 25 °C (Fig. 3 and 4). This may be related to less activity of the proteases at lower temperatures. Our results are in good agreement with one report indicating that the highest amount of anti-thyroid stimulating hormone (TSH) scFv expression could be achieved after induction at 24 °C (Santala and Lamminmäki, 2004). Consistent with our results, based on the design of experiments approach, the optimum temperature for the expression of scFv against CD22 conjugated with apoptin in *E. coli* BL21 (DE3) was also reported as low as 25 °C (Agha Amiri et al., 2017).
Under the above optimal condition (cultivation at 25°C and 0.5 mM IPTG), a high percentage of the target protein was achieved in Rosetta™(DE3) (49% of the total protein). The volumetric productivity of protein reached 403.29 ± 87.50 mg/L (Fig. 4). Conclusively, although at lower temperature there was a general trend for less expression, the temperature effect on protein expression level was not as straightforward as expected.

Conclusion

In this study, for the first time, a scFv from 4D5MOC-B humanized antiEpEX monoclonal antibody was expressed in *E. coli* Rosetta™(DE3) strain. Moreover, here, the effect of cultivation conditions on recombinant antiEpEX-scFv expression level was evaluated. Based on the obtained results, at optimal condition (5 h after induction with 0.5 mM IPTG at 25 °C), the final production yield of the antiEpEX-scFv was 403.29 ± 87.50 µg/mL which makes the subsequent *in vitro* functional trials and structural characterization possible.

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Competing Interests

The authors declare that there is no conflict of interest.

References

Agha Amiri, S., Zarei, N., Enayati, S., Azizi, M., Khalaj, V. and S. Shahhosseini, (2018). "Expression optimization of anti-CD22 scFv-apoptin fusion protein using experimental design methodology." *Iranian Biomedical Journal,* **22**(1): 66–69.

Brischwein, K., Schlereth, B., Guller, B., Steiger, C., Wolf, A., Lutterbuese, R., Offner, S., Locher, M., Urbig, T. and T. Raum, (2006). "MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors." *Molecular Immunology* **43**(8): 1129-1143.

Burges, A., Wimberger, P., Kumper, C., Gorbounova, V., Sommer, H., Schmalfeldt, B., Pfisterer, J., Lichinitser, M., Makhson, A. and V. Moiseyenko, (2007). "Effective relief of malignant ascites in patients with advanced ovarian cancer by a trifunctional anti-EpCAM× anti-CD3 antibody: a phase I/II study." *Clinical Cancer Research*, **13**(13): 3899-3905.

Chen, C., Snedecor, B., Nishihara, J. C., Joly, J. C., Mcfarland, N., Andersen, D. C., Battersby, J. E. and K. M. Champion, (2004). "High- level accumulation of a recombinant antibody fragment in the periplasm of *Escherichia coli* requires a triple- mutant (degP prc spr) host strain." *Biotechnology and Bioengineering*, **85**(5): 463-474.

Choi, T.-J. and T. T. Geletu, (2018). "High level expression and purification of recombinant flounder growth hormone in *E. coli*." *Journal of Genetic Engineering and Biotechnology*, **16**(2): 347-355.

Di Paolo, C., Willuda, J., Kubetzko, S., Lauffer, I., Tschudi, D., Waibel, R., Pluckthun, A., Stahel, R. A. and U. Zangemeister-wittke, (2003). "A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity." *Clinical Cancer Research*, **9**(7): 2837-2848.

Gupta, S. K. and P. Shukla, (2016). "Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications." *Critical Reviews in Biotechnology*, **36**(6): 1089-1098.

Karabulut, S., Tas, F., Tastekin, D., Karabulut, M., Yasasever, C., Ciftci, R., Guveli, M., Fayda, M., Vatansever, S. and M. Serilmez, (2014). "The diagnostic, predictive, and prognostic role of serum epithelial cell adhesion molecule (EpCAM) and vascular cell adhesion molecule-1 (VCAM-1) levels in breast cancer." *Tumor Biology*, **35**(9): 8849-8860.

Kirchner, E., Gerhards, R. and R. Voigtmann, (2002). "Sequential immunochemotherapy and edrecolomab in the adjuvant therapy of breast cancer: reduction of 17-1A-positive disseminated tumour cells." *Annals of Oncology*, **13**(7): 1044-1048.

Kong, B. and G. L. Guo, (2014). "Soluble expression of disulfide bond containing proteins FGF15 and FGF19 in the cytoplasm of *Escherichia coli*." *PloS one*, **9**(1): e85890.

Kong, D., Wang, X., Wang, X., Wang, X., Chen, X., Ji, G., Fu, X. and S. Wang, (2015). "Design, expression and characterization of single chain Fv, Mms13 and the single chain Fv‐mms13 fusion protein." *Molecular Medicine Reports*, **12**(1): 1213-1218.

Larentis, A. L., Argondizzo, A. P. C., Dos Santos Esteves, G., Jessouron, E., Galler, R. and M. A. Medeiros, (2011). "Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage." *Protein Expression and Purification*, **78**(1): 38-47.

Lim, K.-P., Li, H. and S. Nathan, (2004). "Expression and purification of a recombinant scFv towards the exotoxin of the pathogen, *Burkholderia pseudomallei*." *Journal of Microbiology-Seoul,* **42**(2): 126-132.

Rasouli Kery Bozorg, F. and A. Hashemi, (2019). "Efficient expression of EpEX in the cytoplasm of *Escherichia coli* using thioredoxin fusion protein." *Research in Pharmaceutical Sciences*, **14**(6): 554-565

Mack, M., Riethmuller, G. and P. Kufer, (1995). "A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity." *Proceedings of the National Academy of Sciences*, **92**(15): 7021-7025.

Peng, L., Xu, Z., Fang, X., Wang, F., Yang, S. and P. Cen, (2004). "Preferential codons enhancing the expression level of human beta-defensin-2 in recombinant *Escherichia coli*." *Protein and Peptide Letters*, **11**(4): 339-344.

Santala, V. and U. Lamminmäki, (2004). "Production of a biotinylated single-chain antibody fragment in the cytoplasm of *Escherichia coli*." *Journal of Immunological Methods*, **284**(1-2): 165-175.

Schmetzer, O., Moldenhauer, G., Nicolaou, A., Schlag, P., Riesenberg, R. and A. Pezzutto, (2012). "Detection of circulating tumor-associated antigen depends on the domains recognized by the monoclonal antibodies used: N-terminal trimmed EpCAM-levels are much higher than untrimmed forms." *Immunology Letters*, **143**(2): 184-192.

Slouka, C., Kopp, J., Spadiut, O. and C. Herwig, (2019). "Perspectives of inclusion bodies for bio-based products: curse or blessing?" *Applied Microbiology and Biotechnology*, **103**(3): 1143-1153.

Tas, F., Karabulut, S. and D. Duranyildiz, (2014). "Clinical significance of serum epithelial cell adhesion molecule (EPCAM) levels in patients with lung cancer." *Molecular and Cellular Biochemistry*, **396**(1-2): 307-312.

Tsai, W.-C., Wu, T.-C., Chiang, B.-L. and H.-W. Wen, (2017). "Cloning, expression, and purification of recombinant major mango allergen Man i 1 in *Escherichia coli*." *Protein Expression and Purification,* **130**: 35-43.

Wang, M., Zhang, Y., Li, B. and J. Zhu, (2015). "Construction of scFv that bind both fibronectin-binding protein A and clumping factor A of *Stapylococcus aureus*." *Research in Veterinary Science*, **100**: 109-114.

Zheng, P., Sun, X., Guo, L. and J. Shen, (2015). "Cloning, expression, and characterization of an acetolactate synthase (ALS) gene from *Anabaena azotica*." *Process Biochemistry*, **50**(9): 1349-1356.