Effect of Glucose Supplementation and Shaking Speed on the Production of Bioactive Trastuzumab and Chimeric Anti-VEGF Antibody in the Cytoplasm of *Escherichia coli*

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Abstract: Therapeutic antibody market size has been dramatically increasing each year as new target antigens have been identified as well as new type of antibodies are being explored. They are superior than chemical drug as they are highly specific to their co gnate antigen thus can provide very effective medical treatment. Advances in antibody engineering allows for the development of the next-generation of therapeutic antibodies. The ability to produce antibodies in microorganism will further advance this development. Not only in terms of ease of manipulation but also in terms of production cost, which can help increasing accessibility to the patients. In previous study, we investigated the effect of temperature and induction level on the production of full-length humanized anti-Her2 and chimeric anti-VEGF IgGs expressed as cyclonal. In this study, we aim to improve the production yield by investigating the effect of glucose supplementation and shaking speed. When considering glucose addition, it was found that 25 g/L showed significantly improved relative solubility as well as full-length IgG yield for both IgGs with no obvious glucose toxicity. For the shaking speed, it was found that, shaking speed of 150 rpm was the most suitable condition as the relative solubility and relative full-length IgG achieved the highest values for both IgGs among the tested conditions. These conditions can be used as guidelines for fermentation in a bioreactor for further optimization.

Key words: Chimeric anti-VEGF, cyclonal, *Escherichia coli* shuffle T7 express, trastuzumab.

1. Introduction

The global biopharmaceutical market has been expanding continuously with recombinant proteins and therapeutic antibodies representing the main contributors. The market is forecasted to be more than 380 billion dollars in 2024 [1]. Recently, in 2017, the sales of antibody-based medicines became the leading biopharmaceutical reagent with expected sales of 172.8 billion dollars in 2022 representing approximately 20% of the global pharmaceutical market [2]. Interestingly, out of total of 11 therapeutic monoclonal antibodies (mAbs) approved in 2017, the percentage of human and humanized antibodies has dramatically increased. More of next-generation therapeutic antibodies such as bispecific antibodies, antibody-drug conjugates, sugar chain-modified antibodies and low molecular weight antibodies are being developed and

some of them are already in the market [2]. Due to the intrinsic high growth rate of *E. coli*, high cell density cultures are currently used for the production of antibody fragments [3]. With recent advances that allow antibody production in the oxidative cytoplasm of an engineered strain of *Escherichia coli* (*E. coli*) SHuffle strain, production of these next generation antibodies would be easily manipulated and more cost effective than the current method that relies on mammalian cell culture in a bioreactor. Reference [4] showed that the full-length IgG, the traditional mAb format, can be expressed as cytoplasmic IgG, termed 'cyclonal', using this special SHuffle T7 express strain with significant higher yield than the IgG produced in the periplasm of common *E. coli.* Using *E. coli* as a host offers advantage of wide variety of manipulation techniques. For example, simply by replacing variable regions of the scaffold cyclonal with the new variable domains from a different antibody, they could easily create a new IgG molecule with specificity for a new target antigen, illustrating that *E. coli* could be the new workhorse for next-generation antibody production as new genes can be designed, cloned and expressed easily in *E. coli.* We previously reported the construction of the chimeric anti-vascular endothelial growth factor (VEGF) grafting by replacing the variable regions of trastuzumab with the variable region of anti-VEGF. The effect of temperature and induction level on the production of both bioactive trastuzumab and anti-VEGF in SHuffle T7 express were conducted [5]. This strain is based on the *E. coli* B strain that has been designed to promote disulfide bond formation in the cytoplasm that is deficient in proteases Lon and OmpT with chromosomal integration of T7 RNA polymerase gene 1 [6]. The most suitable conditions under the investigated variables, i.e., incubation temperature and induction level, for trastuzumab and anti-VEGF were identified in [5] and these conditions were used as standard in this study to investigate the effect of glucose supplementation and shaking speed.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

E. coli SHuffle T7 express strain (NEB) was used for all experiments in order to express each IgG as cyclonal. SHuffle T7 Express cells containing one of the pMAZ360-cIgG expression vectors were grown in Luria-Bertani (LB) medium, containing 25 μg/ml Spectinomycin and 100 μg/ml Ampicillin, overnight at 37 °C. The next day, overnight cultures were subcultured (1:50 dilution) into fresh LB supplemented with antibiotics and grown at 30 °C until the absorbance at 600 nm reached 0.7 then protein expression was induced by addition of 0.1 mM or 1.0 mM isopropyl b-D-thiogalactopyranoside (IPTG) for trastuzumab and anti-VEGF, respectively. Samples were incubated at 30 °C and cells were harvested 16 hours post induction (hpi).

2.2. SDS-PAGE and Western Blot Analysis

In order to analyze various structures of spontaneous IgG formation, SDS-PAGE analysis was performed under non-reducing conditions. After 16 hours of induction, SHuffle cells were harvested by centrifugation at 5,000 rpm, 4 °C for 10 min. Cell pellets were resuspended in lysis buffer containing 1 mg/mL lysozyme, 50 mM Tris-HCl, 1 mM EDTA and 25 mM NaCl. Branson-SFX 150 (Branson Ultrasonics Co., Danbury, CT) with Micro tips x 4 Branson 1/8 inch (3.2 mm) probe was used to sonicate the suspended cells 3 times for 30 seconds each time. Cell lysates containing soluble proteins were recovered after centrifugation at 13,000 rpm for 10 min to remove cell debris and insoluble proteins. Samples were diluted 1:1 in 2 x Laemmli sample buffer without addition of 2-mercaptoethanol and heated at 100 °C for 10 min. Bradford assay was performed to identify total protein from each sample. Normalized samples were loaded on 10% Tris-HCl gels. Western blotting was performed as previously described [4]. Herceptin or anti-VEGF (mouse/human chimeric Ab) were probed with 1:15,000-diluted anti-human IgG (H+L)-HRP conjugate(Abcam).

2.3. Densitrometric Analysis for Relative Quantification

The densitometric analysis of the protein bands was performed using the program on Bio-Rad ChemiDocTM MP Imaging System (USA). In order to analyze the solubility of each sample, the total IgG of each sample was calculated by addition of all the bands for each sample. For full-length IgG yield, only the band representing constructs with 2 heavy and 2 light chains from each sample was analyzed. Finally, relative solubility and relative full-length IgG yield were calculated by comparing data of each sample with the data from their respective standard conditions, i.e. for trastuzumab, incubation at 30 °C with 0.1 mM IPTG induction was used while, for anti-VEGF, incubation at 30 °C with 1 mM IPTG induction was used. These conditions were used as references because they were reported as the most suitable conditions from the conditions investigated in the previous study [5]. % assembly efficiency for each sample was the ratio of full-length IgG to total IgG.

3. Results and Discussion

3.1. Comparison of Trastuzumab and Anti-Vegf Expressions ss Cyclonal with Glucose Supplementation

Although K12 is the most studied E. coli strain (K strain), E. coli BL21 (B strain) is the most used for recombinant protein production because B strains lack some proteases, achieve higher biomass yields and produces much less acetate than E. coli K12, even in the presence of excess glucose [7]. Optimum amount of carbon source is important to obtaining the desired productivity in the culture process. Enrichment of carbon source using excess glucose in the culture media can result in the reduction in pH due to the acetic acid formation. However, [8] reported that E. coli BL21 (DE3) can tolerate high concentration of acetate, up to 300 mM at pH 6.5 and 7.5. After the effect of temperature and IPTG concentrations were studied in [5], we aimed to investigate the effect of wide range of glucose addition as SHuffle T7 express, the host cell used in our study, is based on E. coli BL21 (DE3) strain. For Trastuzumab, the most suitable condition was expression at 30 °C using 0.1 mM IPTG and, for anti-VEGF, was expression at 30°C using 1 mM IPTG. These conditions were used as standards for comparison of the IgG yield expressed in LB media with glucose supplementation at 5 and 25 g/L of final glucose concentrations. The trend of quantitative analysis of both IgG expressions were similar. Basically, addition of 5 g/L of glucose showed similar relative solubility and relative full-length IgG yield. Interestingly, at 25 g/L glucose addition, although the % assembly efficiency was not affected by high amount of glucose addition but the relative solubility and relative full-length IgG yield were greatly increased, around doubled for trastuzumab, without no obvious glucose toxicity observed as their OD600 value at stationary phase were similar to those of other samples (data not shown). Unfortunately, anti-VEGF expression still showed low production as well as low % assembly efficiency is shown in Fig. 1.

Fed-batch fermentation is more commonly used for the production of recombinant proteins in E. coli. The utilization of feeding strategies is crucial this method as the supply of a nutrient (usually the carbon source) can minimize the production of metabolic by-products that can inhibit growth and product formation. Acetate formation is a common problem when glucose is supplied as a carbon source at high concentrations in the medium. Acetate forms even if the dissolved oxygen concentration is still high, because the specific growth rate is increased when excess glucose is present [9]. In our case, the highest glucose concentration investigated did not have adverse effect for IgG production in SHuffle T7 express strain. Higher amount of glucose as well as the use of alternative carbon sources such as glycerol that do not produce acetate can be further investigated in the future.

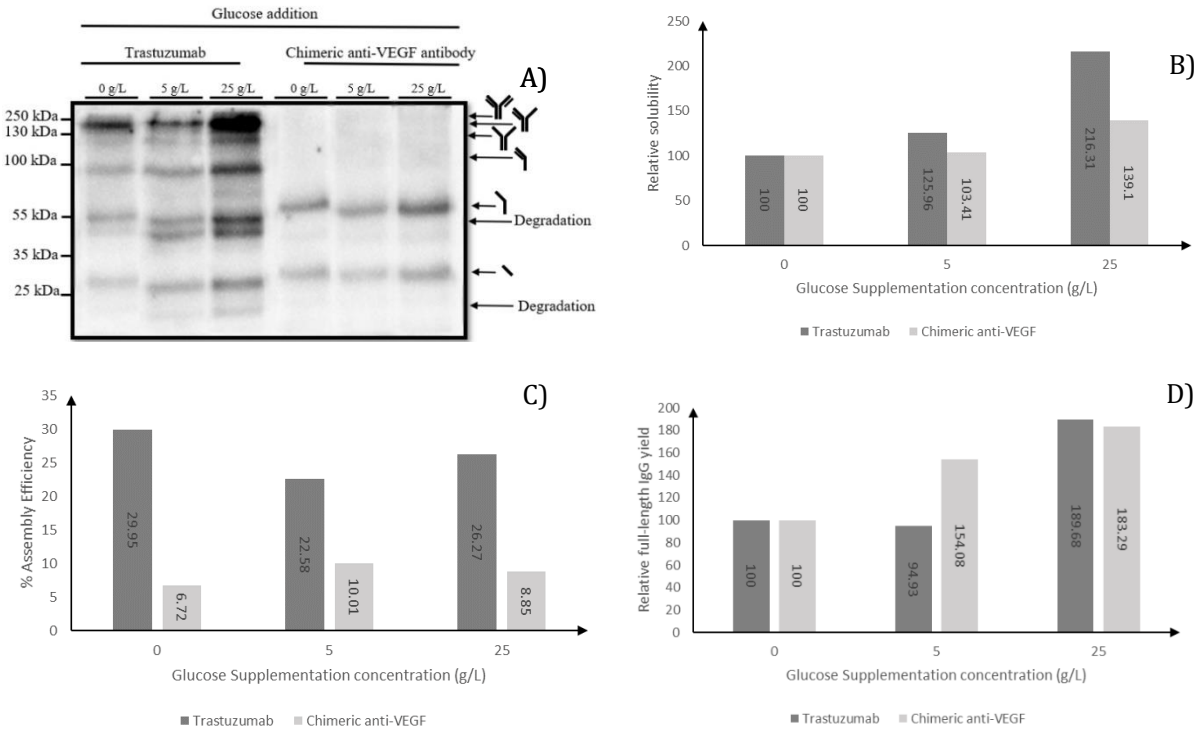

Fig. 1. Analysis of trastuzumab and chimeric anti-VEGF antibody expressions in SHuffle T7 cells with and without glucose supplementation. A) Representative non-reducing western blot of both IgGs expressed at their respective standard conditions compared with expressions in LB media containing glucose supplementation as indicated. Relative quantifications of IgG expressions are shown in B)–D) Band intensities were quantified using Bio-Rad ChemiDocTM MP Imaging System to calculate; B) relative solubility; C) % assembly efficiency, and D) relative full-length IgG yield for both antibodies.

3.2. Comparison of Trastuzumab and Anti-Vegf Expressions ss Cyclonal with Varying Shaking Speed

After we have identified that 25 g/L glucose addition was suitable for culturing of high IgG expression yield for both strains, we aimed to use this condition to optimize the shaking speed. When bacterial culture in liquid media are shaken, cells usually respond with increased growth rate and final cell number as the aeration of the media is increased by increasing of the surface area for oxygen transfer from air to liquid medium. Shaking also reduces localized variations of growth factors, including temperature, pH, media type and presence of nutrients. Regardless of oxygen transfer through shaking, a study has reported that improved homogeneity of growth factors and metabolic products through mixing can also promote cell growth [10]. Different shaking speed can affect not only the dissolved oxygen level in the media but also leakage rate of product as cells can be damaged from vigorous vibration. Therefore, to optimize both oxygen transfer rate while reducing the product leakage into the media, various shaking speeds of the shake flasks were also investigated and the expression analysis is shown in Fig. 2.

In this study, we used shaking speed at 200 rpm as standard condition. We investigated 2 more conditions, which were 150 and 250 rpm shaking speed. A previous research revealed that yield and leakage of antigen-binding fragment (Fab) production of an antibody in E. coli's periplasmic space is dependent on expression strain, culture medium, aeration rate, and the combination of these parameters [11]. At high shaking speed, the protein product may leak from the cells, therefore the results showed decreasing antibody production with increasing shaking speed. Shaking speed lower than 150 rpm was not tested because it is important to consider oxygen transfer rate as well [11]. In our study, we also found that

full-length trastuzumab production in SHuffle T7 express strain showed highest relative solubility and relative full-length IgG yield at 150 rpm and they are decreasing with increasing shaking speed. Similar to what we have observed with glucose supplementation, % assembly efficiency was not affected by shaking speed.

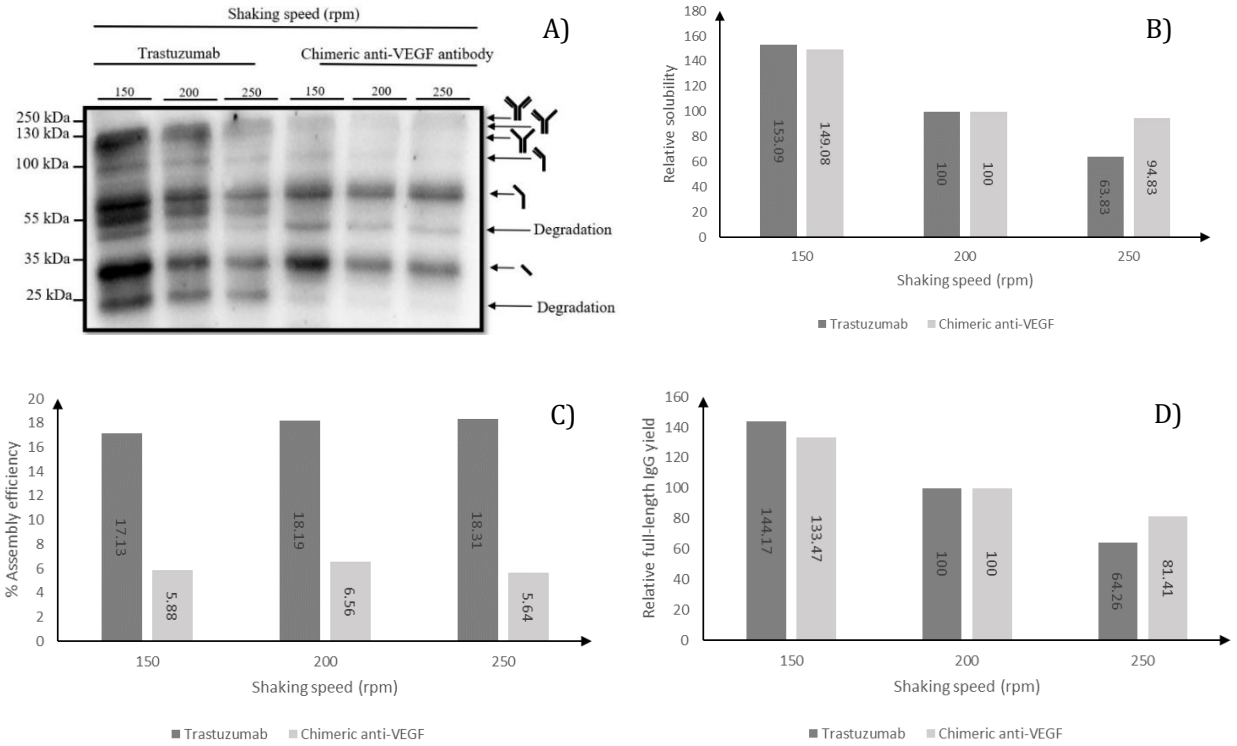

Fig. 2. Analysis of trastuzumab and chimeric anti-VEGF antibody expressions in SHuffle T7 cells at varying shaking speed. A) Representative non-reducing western blot of both IgGs expressed at their respective standard conditions compared with expressions in LB media containing 25 g/L glucose supplementation with varying shaking speed as indicated. Relative quantifications of IgG expressions are shown in B) – D). Band intensities were quantified using Bio-Rad ChemiDocTM MP Imaging System to calculate B) relative solubility, C) % assembly efficiency, and D) relative full-length IgG yield for both antibodies.

As reported by [11], the total Fab yields produced from BL21(DE3) strain, combined yield from periplasmic space and culture medium, increased when shaking speed decreased for 3 out of 4 different Fabs investigated although, depending on the type of medium used and the incubation period, the leakage of Fab into the media were varied significantly. The leakage took place especially during extended cultivation periods. Since our IgG production occurred in the cytoplasm, leakage of the antibody from the periplasmic space was not a problem so we observed the same effect that the total antibody yields increased when shaking speed decreased. Another study reported the attempt to optimize expression of a full-engthIgG antibody in the periplasmic space of E. coli common strain containing reducing cytoplasm [12]. They found that, for multi-polypeptide proteins such as antibodies which are difficult to express in prokaryotic systems due to the complexity of protein folding, by modulating the translation rate, useful quantities of full-length IgG can be expressed and purified from the E. coli laboratory strain HB2151 in standard shaking culture using a simple strategy of reduced inducer concentration combined with delayed induction times. As lowering shaking speed reduces aeration rate, growth rate is reduced as well as the translation rate. This could be the main reason for higher antibody yields observed in [11] and in this study.

4. Conclusion

Most current mAb production utilizes mammalian cells, which allow human-like N-glycosylation, but has several drawbacks associated with bioprocessing and scale-up, resulting in long processing times and high costs. Nonetheless, there are some commercially available therapeutic antibody fragments that are, without glycosylation, still show good antigen binding properties and can be produced in *E. coli*, which are cheaper to cultivate and easy to manipulate. In previous study, using a full-factorial experiment, we identified suitable conditions for trastuzumab and chimeric anti-VEGF, which we used as standards in this study to further optimized the conditions by investigating the effect of glucose supplementation and shaking speed. Since SHuffle T7 express is derived from BL21(DE3) strain, it was found that high glucose concentration supplementation (upto 25 g/L) gave rise to higher IgG yield without cell toxicity thus it is suitable for IgG production. Moreover, lower shaking speed could increased both IgG relative solubility and relative full-length IgG yields. Unfortunately, the assembly efficiency of both IgGs was not effected by any of the factors investigated. Other methods could be investigated in the future. Nonetheless, our studies provided some guildlines for IgG optimization which can be used for fermentation process.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

PC conducted the research; all authors designed the research and analyzed the data; PC and DW-Z wrote the paper; all authors had approved the final version.

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