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Enhanced expression of soluble antibody fragments by low-temperature and overdosing with a nitrogen source

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ABSTRACT

Escherichia coli has been a primary host for the prokaryotic production of antibody fragments (Fabs) and has contributed to several successes in the pharmaceutical industry. Nevertheless, the requirement of disulfide bonds often results in low-yield fermentation and a lack of cost-effectiveness. Despite the improved production of functional Fabs by fermentation below 30 °C, the limited cellular growth needs further work. To address these issues, we investigated the effect of nitrogen supply on the cellular growth and the Fab productivity. We used the anti-human VEGF-A Fab as a model that exhibited poor expression at 37 °C regardless of the amount of nitrogen supplied during fermentation. In stark contrast, the expression yield of soluble Fab with a gross nitrogen supply of 6.91 g/L of broth throughout the fermentation at 25 °C was 332 mg/L. Furthermore, and increased nitrogen supply of 10.9 g/L significantly improved the yield of active form by 59.7% and the cellular growth rate by 39.3%. These results indicate that overdosing of a nitrogen source at low temperature is critical to Fab productivity in *E. coli*.

1. Introduction

Escherichia coli (*E. coli*) is the prokaryotic host most frequently used for basic research and the production of heterologous proteins because it is well characterized physiologically, metabolically, and genetically. Moreover, thanks to the availability of the whole genome sequence and efficient biochemical tools for DNA manipulation, *E. coli* is suitable as a host for the optimized expression of therapeutic proteins that must meet strict quality criteria [1,2].

The antibody, a major class of molecular therapeutics, is a gold standard for immunotherapy with high target specificity. However, the poor tissue penetration and slow clearance of such macromolecules often make them pharmacologically unfavorable for certain clinical applications [3]. An antigen-binding fragment (Fab) consisting of the V_H-C_{H1} domain disulfide-linked to the V_L-C_L domain is approximately one third the size of a native IgG and often more useful as a therapeutic because of its more efficient tissue penetration and mobility [4–6]. Several Fabs have been approved and are under development for the treatment of various diseases [7]. In comparison to a native IgG that requires glycosylation and the complex formation of quaternary structure in mammalian cells, the lack of such modifications on a Fab provides an additional benefit in productivity by bacterial expression in *E. coli,* which features a short generation time and a relatively low production cost [8–10]. Nevertheless, the reducing intracellular environment and fast rate of polypeptide synthesis in *E. coli* often limit the expression of functional Fabs.

To develop an efficient process for Fab production in *E. coli* that achieves a high yield without compromising the Fab's native structure and function, a number of strategies have been investigated [11–13]. One approach involved the co-expression of chaperones with the Fab to facilitate its proper folding into a desired conformation retaining its target-binding activity [14–16]. Alternatively, *in vitro* Fab refolding from inclusion bodies has been demonstrated [17].

However, these strategies suffer from adverse effects such as enhanced proteolysis, increased soluble aggregates, growth inhibition, and lack of scalability, and they are therefore difficult to employ in industrial settings [18]. Control of the growth temperature during fermentation is a facile method that allows the stable expression of heterologous proteins [19,20]. It was previously demonstrated that low-temperature fermentation below 30 °C is advantageous for Fab

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expression in *E. coli,* possibly because the reduced translation rate is favorable for proper Fab folding and alleviates protein aggregation.

Several studies have shown that the yield of a recombinant Fab in *E. coli* was significantly improved at a decreased growth temperature. For example, a 3-fold increase in the yield was observed at 30 °C relative to that at 37 °C, 0.21–0.61 mg/L [19,20]. However, the reduced growth rate and the lower cell density associated with low-temperature fermentation often incur a time and cost burden, restricting its widespread application for Fab expression.

In a quest to mitigate growth retardation while preserving Fab productivity, we chose to increase the amount of total nitrogen supplied throughout low-temperature fermentation in which glucose, a primary factor in growth improvement, was already at saturation.

A recombinant humanized anti-human vascular endothelial growth factor A (VEGF-A) monoclonal antibody fragment (anti VEGF-A Fab) used here as a model Fab, consisting of the light chain (23 kDa) and the heavy chain (25 kDa) interlinked by a disulfide bond and is produced in *E. coli* [22–24]. Anti VEGF-A Fab has been treated for wet Age-related Macular Degeneration (wAMD), macular edema after retinal vein occlusion and diabetic macular edema.

We here report that increasing the supply of the nitrogen source beyond the generally accepted range during the low-temperature expression of model Fab improved both the growth rate and the maximum cell density. Notably, the nitrogen-enhanced growth did not sacrifice productivity but rather improved the expression yield of soluble Fab.

2. Materials and methods

2.1. Host E. coli and expression vector

The *E. coli* W3110 strain was purchased for this study (ATCC, Manassas, VA). A phosphate ion regulation gene was depleted and a valine-resistance gene was restored in W3110 (*ilvG*$^{+/+}$ *ΔphoA*) using the FRT-mediated method for the phosphate depletion expression system and improved growth [25–28].

The Fab expression vector was constructed from the pBR322 vector (New England Biolabs, Ipswich, MA), inserting the Fab expression gene with one phoA promoter [29–31]. The STII signal sequence, the assistance of protein transport to the Sec pathway, was also inserted at the N-terminal sequence of both the light chain and the heavy chain [32–35].

The host cell and expression vector were transformed by electrophoresis (Bio-rad, Hercules, CA).

2.2. Fermentation medium composition

The seed medium prior to the main fermentation [36] was YS medium (5 g/L sodium chloride, 10 g/L yeast extract, 16 g/L soytone). The fermentation for producing Fab was based on 40 g/L initial glucose and 3 g/L potassium dihydrogen phosphate. The initial concentrations of materials containing the nitrogen source were dependent on the experimental conditions (7.4–20 g/L ammonium sulfate, 0.13 g/L ammonium iron citrate, 22.2 g/L yeast extract). The feed consisted of 640 g/L glucose. The concentration of the nitrogen source also varied with the experimental conditions (0–44.5 g/L ammonium sulfate, 20 g/L yeast extract). All materials were manufactured by Merck (Darmstadt, Germany) except the yeast extract and the Soytone (BD, Franklin Lakes, NJ).

The batch medium components were sterilized at 121 °C for 30 min in a 5 L fermenter (Fermentec, Osong, South Korea) and a 50 L fermenter (Sartorius Stedim, Guxhagen, Germany). Tetracycline was used only in the seed medium at 10 μg/mL. It was sterilized by filtration and injected into the seed medium to prevent contamination.

2.3. Calculation of total nitrogen supplied during fermentation (Supplementary Table S1)

The concentration of the nitrogen source added to the broth was calculated by the ratio of molecular weights, i.e., the ratio of the molecular weight of nitrogen to that of ammonium sulfate is 0.21, and to that of ammonium Fe (III) citrate is 0.13. The value was multiplied by the mass of ammonium sulfate added to the medium. In contrast, the ratio of nitrogen to yeast extract was estimated to be 0.11 using the product sheet because calculating the chemical formula of the organic compounds is difficult [37].

The volume of the main medium was 2 L, the feed medium was totally using 0.7 L during fermentation. The pH was adjusted with 200 mL of 14% ammonia solution (Junsei chemical, Tokyo, Japan), and no additional pH regulator was used once the 200 mL of 14% ammonia solution ran out. The 200 mL of 14% ammonia solution was calculated to be equivalent to 11.19 g of nitrogen using the ratio 0.4 of nitrogen to ammonia solution. The antifoam (GE Healthcare, Little Chalfont, UK) went totally 100 mL into fermenter during fermentation for controlling foam, and it did not contain nitrogen. Therefore, the total experimental nitrogen concentrations were calculated by dividing the total nitrogen content by 3 L, which was the total volume at harvest.

The total experimental nitrogen added was 20.73 g of nitrogen (7.4 g/L ammonium sulfate, 0.13 g/L ammonium Fe (III) citrate, and 22.2 g/L yeast extract in the main medium; 20 g/L yeast extract in the feed medium; and 11.19 g in the 14% ammonia solution), 27.93 g of nitrogen (20 g/L ammonium sulfate, 0.13 g/L ammonium Fe (III) citrate, and 22.2 g/L yeast extract in the main medium; 20 g/L yeast extract and 12.6 g/L ammonium sulfate in the feed medium; and 11.19 g in the 14% ammonia solution); or 32.67 g of nitrogen (20 g/L ammonium sulfate, 0.13 g/L ammonium Fe (III) citrate, and 22.2 g/L yeast extract in the main medium; 20 g/L yeast extract and 44.5 g/L ammonium sulfate in the feed medium; and 11.19 g in the 14% ammonia solution). The final calculated experimental nitrogen concentrations were 6.91 g/L, 9.31 g/L, and 10.9 g/L.

In a 50 L fermenter, 9.31 g/L of nitrogen was targeted by using the same materials.

2.4. Fermentation procedure [38]

2.4.1. 5 L fermenter

An *E. coli* stock vial was thawed and inoculated into YS medium in a flask at 37 °C in a shaking incubator. The cell broth was transferred to another flask at one-tenth of the working volume of the second flask after 3 h under the same conditions. To prevent any contamination during the early stages of growth, the fermentation medium and the flasks were sterilized at 121 °C for 30 min before use. The second cell broth was inoculated into the main medium in the 5 L fermenter at one-twentieth of the working volume of the main medium after 4 h.

We used 5 L fermentor produced by Fermentec (Osong, South Korea) which have 1:3 diameter to height ratio, two of 6-blade Rushton impellers and a water-jacketed glass vessel with three baffles. The main fermentations were performed at two different temperatures, 25 °C and 37 °C, and three different nitrogen source concentrations in 5 L fermenters equipped with air and cascaded impeller speed. The air was injected as 2.5 L/min (1.2 vvm) and the impeller speed was adjusted from 300 rpm to 900 rpm in order to get pO_2 as 40%. The pH was controlled to 6.9 with 14% ammonia solution. Each batch fermentation was carried out in duplicate. The feed medium was added at 0.3–0.6 mL/min starting 12 h after inoculation. The culture broth was harvested when the ratio of $OD_{present}$ to OD_{max} approached 80%.

2.4.2. 50 L fermenter

An *E. coli* stock vial was thawed and inoculated into YS medium in a flask at 37 °C in a shaking incubator. Cell broth was transferred to another flask at one-tenth of the working volume of the second flask after