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Comparative Production of Green Fluorescent Protein Under Co-expression of Bacterial Hemoglobin in *Escherichia coli* W3110 Using Different Culture Scales

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Abstract Production of green fluorescent protein (GFP) as a model foreign protein using different culture scales under co-expression of *Vitreoscilla* hemoglobin (VHb) in the industrial *Escherichia coli* strain W3110 (a K12 derivative), was examined. It was found that the VHb co-expressing W3110, exhibited an exceptional and sustained production ability during cell cultures using different scales, while the VHb non-expressing strain showed variable production levels. This high and sustained production ability indicates that the VHb co-expressing *E. coli* W3110, could be successfully employed for practical large-scale production cultures without the need for serious consideration of scale-up problems.

Keywords: culture scale, *Escherichia coli* W3110, green fluorescent protein, *Vitreoscilla* hemoglobin

INTRODUCTION

Vitreoscilla hemoglobin (VHb) has been successfully utilized to enhance production of foreign proteins under microaerobic conditions in several microorganisms, including *Escherichia coli* [1-5]. VHb is known to increase the rate of oxygen usage, especially when dissolved oxygen (DO) is less than 5% of air saturation [1,2]. In our previous work [6,7], it was also shown that co-expression of VHb in *E. coli* enhanced expression of green fluorescent protein (GFP). GFP is a widely used-reporter protein due to several unique attributes; it requires no co-factors or staining for fluorescence, the fluorescence is readily visible from outside the cells, it does not present a large metabolic burden to the host and *in vivo* quantification is possible by simply measuring fluorescence intensity [8-11]. Also employed was the oxygen-dependent *nar* promoter [12,13], for *self-tuning* regulation of VHb expression, due to the natural transition of the dissolved oxygen level for the duration of the culture.

In the present work, GFP production by VHb co-expressing *E. coli* W3110 (a K12 derivative), under the environment of different culture scales, was investigated and the results are compared and discussed.

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MATERIALS AND METHODS

Strains and Plasmids

E. coli W3110 [*F⁻ mcrA mcrB IN(rrnD-rrnE)1 λ⁻*] (ATCC 27325) was used for expressing the GFP and VHb. The recombinant plasmids, pTG that contains the gfp_{uv} gene under a *trc* promoter as a control vector and pTGNV that contains the gfp_{uv} gene under a *trc* promoter and the *vhb* gene under a *nar* promoter, were used [6].

Media and Culture Conditions

M9 minimal medium (12.8 g/L $Na_2HPO_4.7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 mg/L $CaCl_2$ and 1 mM $MgSO_4$) with 5 g/L glucose was used for cell culture. 10 g/L of $NaNO_3$ was added to the medium for induction of the *nar* promoter and 0.1 mM $FeSO_4$ was added as a metal cofactor of VHb protein. Two plasmids pTG and pTGNV were introduced into the *E. coli* W3110. All cultures were performed in M9 medium containing 50 μg/mL ampicillin (Sigma, USA) at 37°C and 250 rpm. Culture experiments for GFP expression were performed in 250-mL flasks with a working volume of 50 mL, 1,000-mL flasks with a working volume of 200 mL, or a 5,000-mL cylindrical glass bioreactor (BioTron, Korea) with a working volume of 2,000 mL. Cultures in the bioreactor were run at 37°C, an agitation rate of 250 rpm and an aeration rate of 1 vvm (air volume/working volume/min).

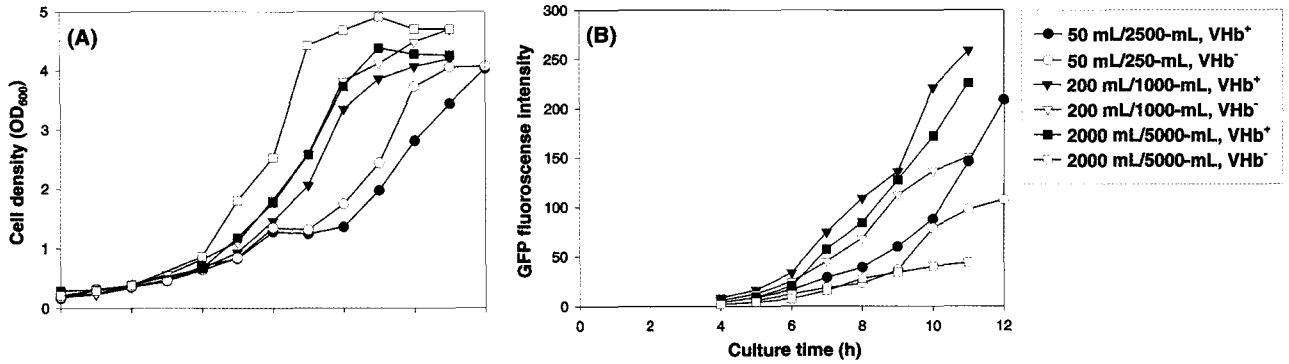

Fig. 1. Time course of cell growth (A) and GFP fluorescence intensity (B) in a 250-mL flask culture with 50 mL of working volume (circle symbols), a 1,000-mL flask culture with 200 mL of working volume (triangle symbols) and a 5,000-mL bioreactor culture with 2,000 mL of working volume (square symbols), under VHb non- and co-expressing conditions. GFP expression was induced by 1 mM IPTG at 5 h for the 50 mL/250-mL culture, 4 h for the 200 mL/1,000-mL culture and 4 h for the 2,000 mL/5,000-mL culture, respectively. Individual duplicate cultures were performed for each scale; average values of the duplicates were reported.

Analytical Methods

Samples were taken at regular intervals from each culture and then measured for optical density at 600 nm (OD_{600}) on a UV-vis spectrophotometer (Shimadzu, Japan). At the mid-growth phase, the cultures were induced to express GFP by the addition of 1 mM (as final concentration) isopropyl β-D-thiogalactopyranoside (IPTG; Sigma). From this induction point, the fluorescence intensity of GFP was also measured using a fluorescence spectrophotometer (Shimadzu).

RESULTS AND DISCUSSION

Several cultures were performed with different scales to investigate the effect of VHb co-expression on cell growth and foreign GFP expression. When cultured in the 50 mL medium in the 250-mL flask (circle symbols), the VHB co-expressing (*vhb*$^+$) *E. coli* W3110 showed a decrease of cell growth (Fig. 1A) but an increase of GFP fluorescence intensity (Fig. 1B), compared to the VHB non-expressing (*vhb*$^-$) strain. This phenomena of decreasing cell growth and increasing production under VHb co-expression, was the same as in the production of another recombinant protein, organophosphorus hydrolase (data not shown). These patterns from VHb co-expression were also identical in the larger-scale cultures; 200 mL of medium in the 1,000-mL flask (triangle symbols in Fig. 1A) and 2,000 mL of medium in the 5,000-mL bioreactor (square symbols in Fig. 1A). Interestingly, when cell growth approached the stationary phase from about 10 h of culture time, the *vhb*$^-$ W3110 showed a gradual reduction of GFP production but the *vhb*$^+$ strain exhibited a peculiar increased pattern of GFP expression (Fig. 1B).

These profiles were also the same in other large-scale cultures of the W3110 strain. These interesting tendencies were not observed using another *E. coli* strain, BL21, that is a B derivative (data not shown). GFP production was reduced during the stationary phase regardless of VHb co-expression. Note that the stationary phase started earlier under larger scale W3110 culture, due to a better initial environment for growth and a subsequent rapid depletion of medium components (recall, M9 minimal medium was used). This occurred around 8 h of culture time in the case of the 200 mL culture in the 1,000-mL flask and around 7 h in the case of the 2,000 mL culture in the 5,000-mL bioreactor.

Specific growth rates (μ) were calculated and depicted for each strain during the exponential growth phase, according to the culture scales, using the least square regression method based on an exponential growth model ($dX/dt = \mu X$; X: cell density, t: culture time) [14] (Fig. 2A). Specific growth rates of the *vhb*$^+$ W3110 were lower than those of the *vhb*$^-$ strain, in any scale of cultures. In addition, the specific growth rates for larger-scale culture were much higher than those of the lower-scale culture. Furthermore, attaining the maximum specific growth rate in larger-scale culture was faster and therefore, cells in larger-scale culture went into the stationary phase more quickly. Also plotted was the specific production rate (q_p) for each strain, based on a cell density-associated production model ($dP/dt = q_p X$; P: product amount) [14] (Fig. 2B). Apparently, the specific GFP production rates of all the *vhb*$^+$ W3110 were higher than those of all the *vhb*$^-$ strain. As described above, specific production rates of the *vhb*$^-$ strain were reduced after entering into the sta tionary phase (about 10 h for the 50 mL/250-mL culture, about 8 h for the 200 mL/1,000-mL culture and about 7 h for the 2,000 mL/5,000-mL culture). However, importantly, the *vhb*$^+$ strain showed a superior production ability even in the stationary phase and had similar values of specific production rates regardless of culture scales. Therefore, it can be surmised that co-expression of VHb confers superior and sustained production ability to the W3110 strain in any culture scale environment.

Similarly the sustained production ability of *vhb*$^+$ W3110 under any culture scales were clearly shown in Fig. 3 when a comparison was made of the GFP fluores-

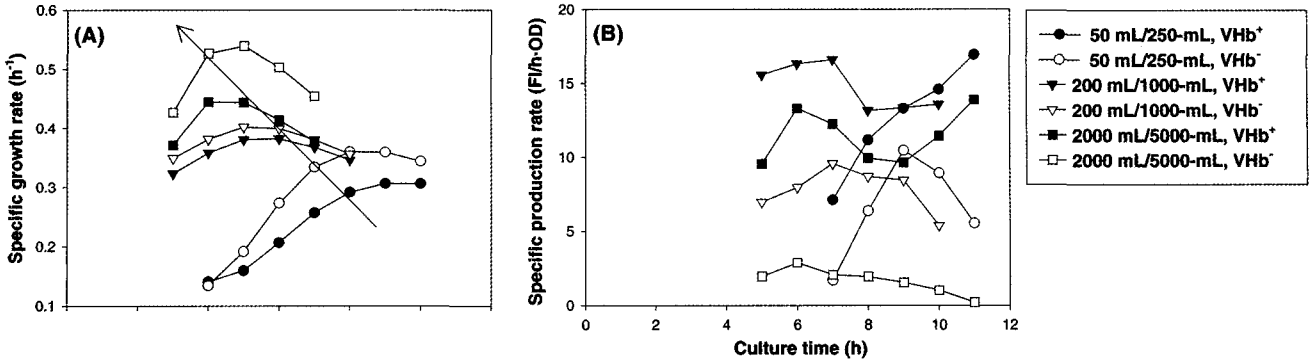

Fig. 2. Specific growth rate (A) and specific GFP production rate (B) in a 250-mL flask culture with 50 mL of working volume (circle symbols), a 1,000-mL flask culture with 200 mL of working volume (triangle symbols) and a 5,000-mL bioreactor culture with 2,000 mL of working volume (square symbols), under VHb non- and co-expressing conditions.

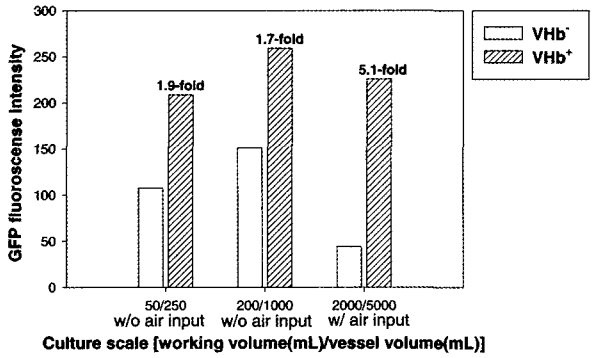

Fig. 3. Comparison of GFP fluorescence intensities at 7 h of culture time, after IPTG induction, according to the culture scales under VHb non- and co-expressing conditions.

cence intensity at 7 h culture time after induction, according to the culture scales. In the case of flask cultures without aeration, it can be regarded that surface aerations were similar throughout the range because the same ratio of working and total vessel volumes were used. Under this environment, VHb showed similar impacts on enhancement of GFP expression (1.7~1.9 fold). In the case of bioreactor culture with aeration (1 vvm), the *vhb*⁻ strain showed lower GFP expression, compared to the flask cultures. This might be due to a low (5 g/L) glucose concentration that could be promptly consumed in culture, especially in a bioreactor culture. Therefore, the fastest cell growth was achieved in a bioreactor culture but the stationary phase that causes highly reduced production ability was also entered quickly (Fig. 2A). Actually, in the case of the bioreactor culture, catabolite repression on GFP expression in the W3110 strain occurred at higher glucose amounts (about 40 g/L) compared to flask cultures (about 20 g/L). This demonstrated that a larger portion of glucose might be used for cell growth in a bioreactor culture (data not shown). While the *vhb*⁻ strain had a lower GFP expression in the bioreactor culture, importantly, higher GFP production (about 5.1-fold) was also achieved in the *vhb*⁺ W3110.

Owing to this superior and sustained production ability that is independent of culture scales, the *vhb*⁺ W3110 strain might be successfully employed for practical large-scale production cultures without the need for serious consideration of scale-up problems.

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