Rapid detection of a gfp-marked *Enterobacter aerogenes* under anaerobic conditions by aerobic fluorescence recovery

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Abstract

A *gfp*- and kanamycin-resistance gene-containing plasmid pUCGK was successfully constructed and transformed into *Enterobacter aerogenes* to develop a rapid GFP-based method for quantifying the bacterial concentration under anaerobic conditions for production of biohydrogen. Since the use of GFP as a molecular reporter is restricted by its requirement for oxygen in the development of the fluorophore, fluorescence detection for the fluorescent *E. aerogenes* grown anaerobically for hydrogen production was performed by developing a method of aerobic fluorescence recovery (AFR) of the anaerobically expressed GFP. By using this AFR method, rapid and non-disruptive cell quantification of *E. aerogenes* by fluorescence density was achieved for analyzing the hydrogen production process.

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Keywords: Anaerobic cultivation; Aerobic cultivation; Biohydrogen; *Enterobacter aerogenes*; GFP; Real time quantification

1. Introduction

*Enterobacter aerogenes* is a facultative anaerobic bacterium that has high potential for fermentative hydrogen production. Even though the theoretical yield of hydrogen production by the NADH pathway in *E. aerogenes* is reported to be as high as 10 mol H₂/mole glucose [1,2], the highest hydrogen conversion rate that has been experimentally reached is only 1.58 mol H₂/mole glucose [1]. Yokoi et al. [3] have reported a hydrogen yield of 2.6 mol H₂/mole glucose using a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*, which indicated that the interaction of *Enterobacter aerogenes* and other hydrogen-producing strains would be beneficial for the production of hydrogen. Furthermore, one of the most promising applications of fermentative hydrogen production will be the combination with wastewater treatment, which is a non-sterile (namely mixed culture) system [4]. Thus, process intensification of a mixed microbial culture by addition of *E. aerogenes* will probably be a potential approach for enhancing hydrogen productivity. Hence, it is indispensable to monitor and control *E. aerogenes* for the augmented hydrogen production.

There are many reporter genes, encoding proteins either with enzymatic activities forming such as colors and luminescence, or with fluorescence, which may be used to trace a targeted microbe. Chemical assay of enzymatic activities encoded in the reporter genes needs the host cells to be disrupted and is time consuming. Luminescence formed by microbial luciferase-catalyzed
reaction has been used for real-time imaging of gene expression in cell cultures [5] and rapid analysis of microbes in a mixed culture [6]. But the light production is closely dependent on the activity of host cells, especially the cofactors of FMNH₂ and NADH, making the luminescence method difficult to be directly used for quantification of the cell concentration in a mixed microbial culture, where the metabolic activity of the targeted microbes will be varied [6,7]. On the contrary, the fluorescence method based on green fluorescent protein (GFP) or its variants has the unique features of real-time detection, non-disruption, non-toxicity to the host cells and requirement of no cofactors, making GFP applicable as both qualitative and quantitative detection methods. A GFP sensor has been first developed to quantitatively monitor GFP-fusion protein production in $E. \text{coli}$ by Albano et al. [8]. The result also indicates that the cell concentration of $E. \text{coli}$ could be quantified by the fluorescence intensity. GFP detection has successfully been used in bioengineering field such as the high cell density cultivation of $E. \text{coli}$ and cell monitoring of yeast [9–12].

But all of the GFP-based methods for cell quantifications mentioned above are used in aerobic culture conditions rather than anaerobic conditions which are required for hydrogen production. The most important obstacle for applying GFP in anaerobic cultures is that mature GFP requires oxygen for the development of the fluorophore to exhibit fluorescence [13]. Few studies have been conducted on the use of GFP in anaerobic cultivation. Scott et al. [14] has found that colonies of the lactic acid bacteria $Lactococcus \text{lactis}$ IL1403 or cells grown in complex anaerobic ecosystems are able to show fluorescence upon subsequent exposure to air. Hansen et al. [15] has reported the lower limit of oxygen concentration for maturation of the GFP fluorophore in $Streptococcus \text{gordonii}$. Phase-contrast and fluorescence microscopy has also been used to distinguish the fluorescent cells in the mixed culture of $Lactococcus \text{lactis}$ [16]. Recently, Verthe et al. [17] has reported the quantification of a gfp-marked $E. \text{aerogenes}$ strain under anaerobic conditions by real-time PCR to study the stability and activity of an $E. \text{aerogenes}$-specific bacteriophage. But until now there has been no report on the rapid quantitative detection of anaerobically-cultivated cells by the fluorescence of GFP.

In this study, we aimed at developing a simple, rapid and quantitative method using GFP for monitoring $E. \text{aerogenes}$ in an anaerobic hydrogen production process. A gfp- and kanamycin-resistance gene (Km')-containing vector, pUCGK, was constructed, and successfully transformed into $E. \text{aerogenes}$. The relationships between the fluorescence intensity of GFP and the cell concentration in aerobic and anaerobic conditions were studied. Aerobic fluorescence recovery (AFR) of GFP was developed to quantify the cell concentration of anaerobically cultivated $E. \text{aerogenes}$ (pUCGK). Feasibility of quantification of $E. \text{aerogenes}$ during the hydrogen production process was also studied.

### 2. Materials and methods

#### 2.1. Bacterial strains and methods

$E. \text{coli}$ JM109 and $E. \text{aerogenes}$ IAM 1183 were used in the present study. Plasmids are listed in Table 1. For genetic manipulation, $E. \text{coli}$ JM109 was grown at 37 °C in Luria broth (LB) (per liter: 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride). For the study of $E. \text{aerogenes}$ IAM 1138, LB and lactose medium (per liter: 15 g lactose, 5 g tryptone, 14 g K₂HPO₄, 6 g KH₂PO₄, 2 g (NH₄)₂SO₄, 1 g sodium citrate, 0.2 g MgSO₄·7·H₂O) were used.

#### 2.2. Plasmid construction

The PCR amplification was done using a Techne Touchgene Gradient Thermal Cycler with 20 or 50 µl system of PCR PreMix (Beijing SBS Genetech Co. Ltd.). Primers were chemically synthesized by Beijing Sunbiotech Co. Ltd. The kanamycin cassette from pET28a was obtained by PCR amplification using primers corresponding to the cloned sequence.

The primers had restriction sites included on the ends to facilitate cloning (forward primer: 5'-CGTTCG-TGGGACCTTTCCATAGGCTCCGCCCCCTGAGAGTC-3' and reverse primer: 5'-GCCGGTACCGGGACCCAGGTGG-CACCTTTTCGGGAAATG-3'). The PCR amplification was done in the conditions of cycle 1: 94 °C, 5 min 45 s; 63.5 °C, 1 min; 72 °C, 1 min; cycles 2-30, 94 °C, 45 s;

### Table 1

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>pQB-2</td>
<td>ampicillin resistance, containing gfp gene</td>
<td>Gift from Prof. Kenji Kohno [18]</td>
</tr>
<tr>
<td>pET28a</td>
<td>Kanamycin resistance</td>
<td>Novagen 69864-3</td>
</tr>
<tr>
<td>pUC18</td>
<td>ampicillin resistance, lac promoter</td>
<td>GenBank, L09136</td>
</tr>
<tr>
<td>pUC18</td>
<td>Kanamycin resistance, pUC18 containing Km' cassette from pET28a</td>
<td>This work</td>
</tr>
<tr>
<td>pUCGK</td>
<td>Kanamycin resistance, pUCG containing Km' cassette from pET28a</td>
<td>This work</td>
</tr>
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</table>
The Km\textsuperscript{r} cassette was first inserted into pUC18 between [19]. For selection of the transformant of were based on the procedures in Molecular cloning procedure of the manufacturer. Other DNA manipulations were: cycle 1: 94°C, 5 min 45 s; 54°C, 1 min; 72°C, 1 min; cycles 2–30, 94°C, 45 s; 54°C, 1 min; 72°C, 1 min with a final elongation step of 5 min.

Restriction and other DNA modifying enzymes were obtained from Takara Co. Ltd or NEB. Restriction enzyme digestions were performed according to the procedures in Molecular cloning [19]. For selection of the transformant of E. aerogenes, the Km\textsuperscript{r} cassette was first inserted into pUC18 between two AvaII sites to form pUCK. The gfp gene was then inserted into the pUCK between the sites of EcoRI and KpnI to form pUCGK (Fig. 1).

2.3. Bacterial transformation

Competent E. coli and E. aerogenes cells were prepared using the CaCl\textsubscript{2} method, and they were transformed according to standard procedures [19]. Successful transformants were identified by plating on the appropriate antibiotic-containing media (for E. coli, 100 µg/ml ampicillin or 30 µg/ml kanamycin; for E. aerogenes, 30 µg/ml kanamycin was supplemented in LB medium).

2.4. GFP expression and fluorescence observation

For the expression of GFP in the transformed cells, IPTG was added at a final concentration of 1 mM before the cultivation in LB. However, in the lactose medium, GFP could be expressed without the addition of IPTG as the inducer. The fluorescence of GFP-expressing cells was assessed by using a fluorescence microscope (Nikon E600, Japan) fitted with a Ba-2A filter set (excitation 450–490 nm).

2.5. Bacterial cultivation

All cultivations of E. aerogenes (pUCGK) were carried out on a reciprocal shaker at 150 rpm and 37°C. For the aerobic cultivation, a 500-ml Erlenmeyer flask containing 100 ml of LB medium (IPTG was added at a final concentration of 1 mM before the cultivation) or lactose medium was inoculated with a 1-d seeding culture at an inoculum size of 1% (v/v).

For the anaerobic cultivation of E. aerogenes (pUCGK), a 100-ml anaerobic bottom containing 40 ml of lactose medium was previously degassed to anaerobic conditions (sparking with nitrogen gas prior to tubing, head-gas replacement and then autoclaving), and was subsequently inoculated with a 1-d seeding culture at an inoculum size of 2.4% (v/v).

2.6. Aerobic fluorescence recovery method

40 ml of culture broth at different culture times were first centrifuged at 10 200 × g for 10 min. The centrifuged cells were resuspended in 40 ml of PBS buffer (pH 7.4) and centrifuged under the same conditions. This process was repeated two times to wash out the original broth. Then the cells were resuspended in 40 ml of PBS buffer and transferred into an unsealed flask of 50 ml to be cultivated on a reciprocal shaker at 150 rpm and 37°C. The fluorescence intensity of the samples obtained from the flask was measured every 10 min. To study the influence of oxygen concentration on AFR, different ways for oxygen supply were used: (1) in an unsealed flask without any treatment; (2) aeration in an unsealed flask to attain a high oxygen concentration; (3) N\textsubscript{2} supply in a sealed flask to attain a low oxygen concentration.

2.7. Analytical methods

During cultivation, the total amount of gas produced was measured with a measuring cylinder. The optical density at 600 nm (OD\textsubscript{600}) for evaluating the cell concentration was measured with a spectrophotometer (Shimadzu UV-1206, Japan) with an error of less than 1%. The components of gas evolved were analyzed using gas chromatographs (Shimadzu GC8A, Japan) with a Parapak Q 80–100 mesh column and a TCD detector. For the measurement of fluorescence intensity, a fluorescence spectrophotometer (Hitachi F-2500, Japan) was used with the parameters of excitation at 488 nm and
emission at 510 nm. The relative error for fluorescence detection was 3.37%. To examine the GFP expression under anaerobic conditions, SDS–PAGE was performed by the standard method [18].

3. Results

3.1. Plasmid construction, transformation and GFP expression under aerobic conditions

_E. aerogenes_ IAM 1138 was found to have a specific resistance to ampicillin. It can survive in LB with up to 200 µg/ml ampicillin, while it has no resistance to kanamycin. Thus, a kanamycin-resistance gene (Km') cassette from pET28a was inserted into two AvaII sites of pUC18 that were originally located in the sequence of the ampicillin-resistance gene to form a Km'-harboring pUCK. Consequently, a vector of pUCGK was constructed in which the _gfp_ gene was placed downstream of the _lac_ promoter as described in Section 2. In this work, an enhanced mutant of _gfp_ from pQB-2 was used. Transformants of _E. aerogenes_ were successfully obtained on kanamycin-containing media (30 µg/ml). Fluorescence resulting from the transformants harboring pUCGK could be easily observed by a fluorescence microscope.

As microscopically observed under normal optical and fluorescence conditions, all the bacterial cells uniformly showed a green color after the excitation. The fluorescence spectrum showed the typical GFP expression in _E. aerogenes_ (pUCGK), i.e., an emission peak at 513 nm with the excitation at 488 nm (data not shown).

Fig. 2 shows the profile of cell optical density and fluorescence intensity during the whole course of cultivation in lactose medium under aerobic conditions. The fluorescence of the ultrasonically-disrupted bacterial solutions of the PBS-resuspended cells was lower than its supernate because of the light-scattering effect of the cell debris. The values of the other two cases with whole cells were much lower than the respective cases by cell disruption, indicating that the cell wall acted as a barrier to fluorescence transmission. But all of the four curves of fluorescence intensity had the same trends during cultivation as shown in Fig. 2, which indicated that the four ways of measuring fluorescence intensity would give the same trend. To eliminate the effect of the medium components on GFP fluorescence, only the fluorescence intensity of the cells resuspended in PBS buffer was used for the quantification of the cell concentration in this paper. Some studies have shown that GFP expressed in host cells needs at least 95-min for maturation [20], which was also observed in the present

Fig. 2. Profile of cell density and fluorescence intensity during the cultivation in lactose medium under aerobic conditions. Symbols: □, OD600; ●, fluorescence intensity of the culture broth; △, fluorescence intensity of the cell resuspension in PBS buffer; ○, fluorescence intensity of the cell resuspension after ultrasonication treatment; ■, fluorescence intensity of the supernatant of the cell solution treated by ultrasonication.

Fig. 3. (a) A profile of fluorescence intensity during the AFR process after exposure to the air. OD600 = 0.31 (□); OD600 = 0.155, (●); OD600 = 0.0775 (△); OD600 = 0.03875 (○). (b) Relationship between fluorescence recovery rate, maximum fluorescence intensity after AFR with cell concentration. Fluorescence recovery rate (□). Maximum relative fluorescence intensity after AFR (●).
study (Fig. 2). Within the period of 4–10 h, the fluorescence intensity had a good linear relationship with the optical density (data not shown).

3.2. Aerobic fluorescence recovery of GFP-harboring strain cultivated under anaerobic conditions

SDS-PAGE results indicated that the GFP could also be expressed in *E. aerogenes* (pUCGK) under anaerobic cultivation, but it was a non-fluorescent form (data not shown). An experiment was thus designed to examine the possibility of AFR for the non-fluorescent intermediate expressed under anaerobic conditions. Fig. 3(a) shows the profile of fluorescence intensity of the anaerobic cultures at different cell concentrations after exposure to the air. During the whole course of incubation in PBS buffer, the optical density was constant, while the fluorescence intensity was increased with the aerobic recovery time, indicating the feasibility of AFR for regeneration of GFP fluorescence in the bacterium cultivated anaerobically. Different oxygen concentrations supplied by different ways were also examined for AFR, which gave the same fluorescence recovery curve (data not shown). Thus, oxygen transferring and its concentration were not the key factors for AFR.

As all of the curves in Fig. 3(a) exhibited a first-order reaction, which had a similar half time of 15–16 min, the AFR process of the anaerobic cells can be expressed as:

\[ -\frac{d[F^*]}{dt} = \frac{d[F]}{dt} = k[F], \]

where \( F^* \) represents the non-fluorescent intermediate, \( F \) represents the fluorescent protein of matured GFP, proportional to fluorescence intensity. Assuming that the GFP expression per cell concentration (\( X \)) is constant, then Eq. (2) can be obtained:

\[ \frac{[F^*]}{X} = a. \]  

When all the non-fluorescent intermediates are matured by reacting with oxygen to form the matured GFP in a short time, then the following boundary conditions can be obtained:

\[ t = 0, \quad [F^*] = [F^*_0], \quad [F] = 0, \]  

\[ t = \infty, \quad [F^*] = 0, \quad [F] = [F]_{\text{max}}. \]  

Fig. 4. (a) Profile of cell optical density, fluorescence intensity, total gas volume and pH during the whole course of anaerobic cultivation. Optical density (\( \square \)); maximum relative fluorescence intensity (\( \bullet \)); relative fluorescence recovery rate (\( \triangledown \)); total gas volume (\( \triangle \)); pH (\( \circ \)). (b) Relationship between fluorescence recovery rate, maximum fluorescence intensity after AFR with cell concentration during the anaerobic cultivation from 4 to 10 h. Fluorescence recovery rate (\( \blacktriangle \)). Maximum relative fluorescence intensity after AFR (\( \blacklozenge \)).
For cells cultivated for a certain time under anaerobic conditions, $[F]_{\text{max}}$ is dependent upon the amount of non-fluorescent intermediate already synthesized inside the cells, $[F]_0$. Since the change in cell concentration in the AFR experiment can be ignored, from the above equations, the following equations can be obtained:

$$[F]_{\text{max}} = aX,$$  \hspace{1cm} (5)

$$\Delta [F]/\Delta t = kaX.$$  \hspace{1cm} (6)

Eqs. (4) and (5) show that both the maximal fluorescence intensity and the fluorescence recovery rate have linear relationships with the GFP-harboring cell concentration (OD$_{600}$ in this paper).

Fig. 3(b) shows the dependency of the maximal fluorescence intensity after the AFR, as well as the recovery rate before the intensity reached the maximal on the optical density of cells (OD$_{600}$), which agreed well with the above theoretical consideration. The relative errors for fluorescence intensity and the fluorescence recovery rate by AFR were less than 4% and 5%, respectively, in triplicate measurements. The relationships of $\Delta [F]/\Delta t = 93.7 \times$ OD$_{600}$ and $[F]_{\text{max}} = 162.9 \times$ OD$_{600}$ were drawn from Fig. 3(b). Thus, we can conclude that the aerobic fluorescence recovery method developed in the present study can be used to quantify the GFP-harboring cell concentration perfectly under anaerobic culture conditions.

### 3.3. Quantification of cell concentration during anaerobic cultivation by the AFR

Fig. 4(a) shows the profile of cell optical density, fluorescence intensity, pH and total gas volume during the whole course of cultivation in lactose medium under anaerobic conditions. After 22 h cultivation, the final hydrogen volume reached 675 mL H$_2$/L culture broth. Both the maximum fluorescence intensity and the fluorescence recovery rate obtained by the AFR showed the same trends during the whole course of cultivation. Within 4–10 h, the two parameters of the AFR had a good linear relationship with the optical density (Fig. 4(b)), which gave the results of $\Delta [F]/\Delta t = 360 \times$ OD$_{600}$ − 50 and $[F]_{\text{max}} = 401 \times$ OD$_{600}$ − 44. Thus, the cell concentration during the anaerobic cultivation could be rapidly quantified by the AFR, just similar to the quantification by GFP detection in aerobic conditions. But after 10 h, the two parameters of the AFR decreased, showing a different trend with optical density and total gas volume. GFP concentration per ml culture broth measured by SDS–PAGE decreased with the culture time after the AFR rate and maximum AFR value reached their peaks, agreeing well with the results of the AFR detection during the whole course of cultivation (data not shown).

### 4. Discussion

Quantification of the amount/activity of a microbial strain in a bioprocess are of particular importance from the view-point of process control or optimization. GFP has been successfully used in evaluation of many kinds of microbes, mainly under aerobic conditions. Until now, there are two papers reporting the use of GFP in Enterobacter aerogenes. Fu et al. [21] used a self-constructed plasmid pMMBGFPβ to prepare a prey strain of E. aerogenes for protistan grazing analysis by using flow cytometry. But the GFP expression has been performed under the aerobic condition. Another work has been done by Verthe et al., in which gfp gene was inserted into the chromosome of E. aerogenes BE1 [17]. While in their study, a real-time PCR was used to quantify gfp gene. Compared with the direct detection of GFP fluorescence, PCR may be time-consuming and dependent on the cell disruption. Thus, in our study, we tried to develop a method of AFR by simple fluorescence detection to quantify E. aerogenes IAM1183 rapidly and non-disruptively.

As described above, the expression of GFP molecules under the anaerobic cultivation conditions can be confirmed by SDS–PAGE. However, to exhibit the fluorescence of a mature GFP molecule, the chromophore in the GFP intermediate which is naturally formed by an autocatalytic cyclization of the tripeptide, -Ser-65-Tyr-66-Gly-67-, in the primary structure must be followed by air oxidation to the formation of an exo-methylene double bond [13]. But under anaerobic condition, no oxygen is available for the maturation of the GFP chromophore. The works reported by Scott et al. [14] and Hansen et al. [15] imply that air exposure make the anaerobically-expressed GFP intermediate to be fluorescent. Based on these findings, we focused on quantitative analysis of anaerobic cells by fluorescence detection of AFR in the present paper. The AFR process at different cell concentrations exhibited a same half time of 15–16 min. This half time was much smaller than the reported 76 min in the pure GFP oxidation process [13], which would most probably be due to the trace oxygen in the cultivation system that had rapidly reacted with the non-fluorescent intermediate GFP. Moreover, the AFR of the whole cells exhibited a first-order reaction, which was identical with the pure GFP oxidation process and formed theoretical basis of the AFR method. As shown in Fig. 4(a), using the fluorescence recovery rate, the detection can be done in a short time of less than 10 min without the cell disruption.

Even though the AFR method was successfully developed for cell quantification in an anaerobic bioprocess, it was not suitable for the cells after 10 h cultivation as shown in Fig. 4(a), which was the same as that of the
aerobic cultivation in the same lactose medium (Fig. 2). This could mainly due to the stability of GFP in low pH value. As shown in Fig. 4(a), the pH value of the culture broth decreased with the cultivation time, reaching pH 6.5 after 10 h. The pH profile in Fig. 2 showed a similar trend as that in Fig. 4(a). The pH stability of the GFP in intact cells was studied by resuspending the aerobic cultured cells with strong GFP fluorescence in buffers with different pH (3.8–7.4). As a result, GFP in the cells was stable in the pH of 6.5–7.4, but the intensity was reduced significantly in the pH of 3.8–6.5 (data not shown). The stability of GFP in the intact cells was similar to that in the cell extract obtained by cell disruption in another experiment, which is also identical with that of the pure GFP reported by Ward [22]. The decrease in the culture pH with the culture time and the instability of GFP at the low pH under 6.5 has thus resulted in the decrease of fluorescence intensity of GFP after 10 h anaerobic cultivation (Fig. 4(a)). This could also be verified by another experiment in LB medium with the induction of IPTG where the final pH value was above 6.5 and a good linear relationship between the optical density and fluorescence intensity was observed during the whole cultivation course (data not shown). Incidentally, the decrease in GFP concentration after 10 h anaerobic cultivation as confirmed by SDS-PAGE analysis described above suggests that the instability of GFP under low pH may be caused by its degradation. For the anaerobic cultivation of E. aerogenes (pUCGK) for hydrogen production, this problem could be solved easily by pH control.

To examine the plasmid stability of PUCGK in E. aerogenes, bacterial subcultures with and without the presence of the antibiotics were carried out. Every subculture was performed for 12 h, and after one subculture, the bacterial cells were inoculated into the new media for the subsequent cultivation. After 5 generations of the subcultures, only 4% of total cells without the addition of the antibiotics lost the plasmid (data not shown). This result shows that the plasmid of PUCGK is stable in E. aerogenes. Since the inoculums used in the cultures in Figs. 2 and 4(a) were within twice precultures with addition of antibiotics (kanamycin), the plasmid stability was also not the reason which might have caused the decrease of fluorescence by AFR (Fig. 4(a)). In addition, for the further wide use of this AFR method to anaerobic cultivation, inserting gfp into the chromosome of E. aerogenes without induction for gene expression is also necessary.

In summary, by using this AFR method, rapid and non-disruptive cell quantification of E. aerogenes under anaerobic cultivation was achieved. The AFR method discussed above shed light on the quantification of gfp-harbouring E. aerogenes or E. aerogenes with fusion protein of GFP-hydrogenase for effective hydrogen production under the anaerobic conditions.

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