Assessment of Fluorescence Intensity of Green Fluorescent Protein in *Escherichia coli* under Low pH Conditions

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Article history: Received 20 May 2013, Received in revised form 25 June 2013, Accepted 30 June 2013, Published 8 July 2013.

**Abstract:** Application of green fluorescent protein (GFP) as a molecular biosensor or reporter is limited by several factors especially its poor fluorescence at low pH conditions. There are conflicting data on this limitation, however, systematic studies to assess the importance of this factor for growing bacterial cultures are lacking. In this study, effect of low pH on GFP green and red fluorescence intensities was demonstrated in batch cultures of *Escherichia coli* SCC1. The bacterial cells that express GFP were grown and photoactivated with blue light which made them absorb green light and emit red. In order to assess the fluorescence intensity under different pH conditions, relative fluorescence (RF) (absolute fluorescence/OD$_{650}$) and percentage red-fluorescent to green (R/G (%)) were determined aerobically and anaerobically. The green and red fluorescence intensities were found to be reduced in pH values 4.0 and 5.0. The GFP green fluorescence has very low intensity of less than 70 RFU at pH 5.0 and about 20 RFU at pH 4.0. The intensity was shown to be higher (up to 110 RFU) at pH 6 - 7 in the aerobic cultures. It was shown that GFP red fluorescence changes with time in different pH conditions both aerobically and anaerobically. The red fluorescence intensity has higher magnitude anaerobically compared to the aerobic cultures. Thus, at pH levels 4 - 5, the red and green fluorescence intensities were both found to be significantly reduced.

**Keywords:** green fluorescent protein; *Escherichia coli*; pH; fluorescence.
1. Introduction

In the last decade, novel molecular biology techniques based on green fluorescent protein (GFP) (named by Morin and Hastings (1971)) have been discovered. The GFP originated from jellyfish Aequorea victoria (Shimomura et al., 1962). Modified variants of this GFP that possess enhanced fluorescent intensity and improved spectral features (Heim and Tsien, 1996) have been observed. Since from the first cloning of gfp-gene, the A. victoria GFP and its variants became powerful tools in cell biology that play vital roles as molecular signals for rapid quantification and monitoring of parameters in bioprocesses.

These fluorescent techniques employing GFP and its variants have distinctive characteristics of real time detection, safe to the host cells and do not require any cofactor (Zhang et al., 2005). The technical revolution behind green fluorescent protein discovery was related to a chromophore property which is the source of its fluorescence (Shimomura et al., 1962). A tripeptide motif within the GFP primary structure forms the chromophore, and its fluorescence turns on automatically in any living system that expressed it (Cody et al., 1993). Thus, the chromophore maturation in the GFP does not require any auxiliary factor or enzyme but depends only on molecular oxygen (Tsien, 1998).

The minimum level of pH (6.0) at which the intensity of GFP fluorescence is unaffected in Streptococcus gordonii culture was reported by Hansen et al. (2001). The pH values lower than 6.0 cause significant drop in the intensity of GFP fluorescence (Scott et al., 1998), but when re-suspended in phosphate buffer solution (PBS, pH 7.4), the acid induced decrease in the intensity of the fluorescence was reversible (Hansen et al., 2001).

The aims of this study were (i) to express the GFP in a Gram-negative bacterium; E. coli which is a model organism used in many bioprocesses; (ii) to test the limits of the use of GFP as a marker under suboptimal conditions of low pH, then address these limitations in a practical application of GFP as a molecular marker in the Gram-negative bacterium E. coli SCC1 grown in batch cultures under aerobic and anaerobic conditions; and (iii) to determine which growth conditions allow sufficient GFP reporter signals to be useful in connection with in vitro as well as in situ studies.

2. Materials and Methods

2.1. Bacterial Strain

The E. coli SCC1 (containing gfp gene in its chromosome (Miao et al., 2009)) was used in this research and was obtained from Biochemical Engineering unit of the School of Chemical Engineering, University of Birmingham, UK.
2.2. The Media

Lennox broth (LB) and nutrient agar (NA) were used. LB contains 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl and distilled water. The 28 g/L of NA (Oxoid) was dissolved in distilled water and used to prepare agar plates for bacterial colonies.

All the prepared media were sterilized before use in an autoclave (Series 300, LTE Scientific Ltd; Greenfield) at 121 °C for 15 min.

2.3. Glucose Stock Solution

The 40% (w/v) glucose stock solution was prepared and sterilized separately at 121 °C for 5 min.

2.4. Bacterial Cultivation in Buffer Solutions

To investigate the effect of pH on the intensity of GFP fluorescence, phosphate-citrate buffers; pH 4.0, 5.0, 6.0 and 7.0 were prepared according to the method of McIlvaine (1921). The bacterial cells were grown aerobically to an OD$_{650}$ of about 1.0 in 250 mL conical flasks (duplicate) containing LB medium supplemented with 0.4% (w/v) glucose. The 1 mL of the culture was harvested into each of 16 Eppendorf tubes and centrifuged. The supernatant was discarded. The cells from four (4) sets of Eppendorf tubes were carefully re-suspended in 1 mL (each) of one pH condition. Two from each set were transferred into two test tubes containing 9 mL each of the buffer with the corresponding pH condition (i.e. two test tubes each for pH 4.0, 5.0, 6.0 and 7.0) and the other two into two 50 mL conical flasks (also two for each pH condition and contain 9 mL each of the buffer with that pH). The cultures in the test tubes were incubated at 37 °C without shaking for 4 h while those of the flasks were incubated in the incubator-shaker (mentioned earlier) at 37 °C and 200 rpm also for 4 h. Culture samples were collected hourly and the intensities of the green and red fluorescence were analyzed.

2.5. Analytical methods

The OD$_{650}$ measurements for biomass evaluation were carried out by using spectrophotometer (Uvikon-922, Kontron-Instruments; Buckinghamshire, England) and growth-rates were calculated.

The culture fluorescence measurements were performed with fluorescence spectrophotometer (LS50B model, Perkin-Elmer Ltd, UK) with Slit-Width at 7.5 nm (excitation) and 15.0 nm (emission), and Integrate-Time (IT) at 5 sec. Green and red fluorescence were measured respectively at 480 nm/510 nm (excitation/emission) and 525 nm/600 nm (excitation/emission). Relative fluorescence (RF) (i.e. absolute fluorescence/OD$_{650}$) and percentage of red-fluorescent to green (R/G (%)) were
Measurement of pH was carried out using pH-meter (SevenEasy, Mettler-Toledo, Switzerland). Buffers of pH 4.0, 7.0 and 10.0 were used to calibrate the pH-meter.

3. Results and Discussion

In Fig. 1, it has been shown that, both aerobically and anaerobically, the GFP green fluorescence has very low intensity of less than 70 RFU at pH 5.0 and about 20 RFU at pH 4.0. The intensity was shown to be higher (up to 110 RFU) at pH 6-7 in the aerobic cultures. In Fig. 2, it was indicated that GFP red fluorescence changes with time in different pH conditions both aerobically and anaerobically. Thus, at pH levels 4 and 5, the red and green fluorescence intensities were both found to be significantly reduced with lower intensities at pH 4 for the aerobic cultures (Figs. 1 and 2).

![Figure 1](image-url)

**Figure 1.** Effect of pH on the relative green fluorescence intensity under aerobic and anaerobic conditions. O2, aerobic conditions; Ø2, anaerobic conditions; RFU, relative fluorescence unit. The error bars are the standard deviations.

Because of the reduction effect of lower pH values on the GFP red and green fluorescence intensities, the %R/G ratio was directly affected. Fig. 3 shows an increase in the magnitude of %R/G at lower pH values for both aerobic and anaerobic cultures.
Figure 2. Effect of pH on the relative red fluorescence intensity under aerobic and anaerobic conditions. $O_2$, aerobic conditions; $\varnothing_2$, anaerobic conditions; RFU, relative fluorescence unit. The error bars are the standard deviations.

Figure 3. Effect of pH on the percentage red-fluorescence to green ($%R/G$) under aerobic and anaerobic conditions. The error bars are the standard deviations.

Takahashi and Sato (2010) investigated the effect of pH change on red fluorescence. In their finding, the R/G values have not significantly change at pH levels between 6 and 8 at 36 °C. In this
research, it was found that the red fluorescence intensities change significantly with time at pH values 4 - 5. Thus, both the red and the green fluorescence intensities tend to decrease in low pH values investigated (pH 4 and 5) (Figs. 1 and 2). Since the GFP red and green fluorescence intensities were both affected by low pH, this effect has directly affected the %R/G ratios by increasing the magnitudes. This is the reason why in Fig.3 there was higher %R/G levels in pH 4.0 in both aerobic (up to about 1%) and anaerobic (up to about 3.5%) cultures compared to the higher pH levels (pH 6 and 7).

4. Conclusions

The in vivo analyses in this study demonstrate that, this reporter protein (GFP) can indeed be used effectively for monitoring, assessing or measuring many cellular activities in bioprocesses. A low pH in the environment results in low fluorescence yield, and for fermentative organisms, it is highly important to control the external pH especially within the range of 6 - 7.

References


