Use of Fluorescent Protein Fusions to Optimize Membrane Protein Expression in Anaerobic Photoheterotrophic *Rhodobacter sphaeroides*

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**Summary**

*Rhodobacter sphaeroides* generates abundant amount of intracellular membranes (ICM) hosting photosynthetic apparatus under anaerobic photoheterotrophic growth conditions rendering it an attractive organism for membrane protein expression. We use native photosynthetic promoters to drive the membrane protein expression in *Rhodobacter*, which helps synchronizing the synthesis of ICM with the protein expression to maximize the efficiency of the expression. However, *Rhodobacter* is not a well-established expression system unlike other prokaryotic expression systems, e.g. E.coli and a myriad of experimental parameters still needs to be explored such as mode of growth, growth medium and temperature, duration of culturing, as well as promoter induction conditions. Conventional methods for protein expression screening, e.g. western-blotting, are labor intensive, time consuming and unsuitable for real-time expression screening.

Here we describe the use of fluorescent protein fusions as a method for optimizing and accurately quantifying real-time membrane protein expression in *Rhodobacter sphaeroides* under anaerobic photo heterotroph growth conditions. The culture growth was driven by high power infrared LEDs with a light intensity of 4W/m². We used a homologous membrane protein (a novel aquaporin water channel protein, RsAqpZ) and human proteins, occludin and AQP9, to determine the optimum culturing temperature for the protein expression. A robust correlation between fluorescence intensity and Western-blot densitometry was established using mBanana fluorescence protein. RsAqpZ was expressed best at 34°C (optimum growth temperature of *Rhodobacter*) reaching a titer of 20 mg/L, while occludin and AQP9 had the highest expression level at 25°C with titers 7.5 and 0.5 mg/L, respectively.

**Introduction**

Membrane proteins (MP) are a difficult class of proteins to study, since the majority of the proteomic tools to date are designed for soluble proteins. However, MPs comprise approximately 30% of the total genetic material as evidenced by the investigation of the available full genomes sequences and they have a high medical as well as biotechnical importance [1]. These crucial features of MP make them compelling to address the associated challenges. The difficulties with expression of MPs have been reported in number of other protein expression systems such as *Escherichia coli*, yeast and mammalian cell culture [2]. The anaerobic photoheterotroph, *Rhodobacter sphaeroides*, has the desirable characteristic of large quantities of intracellular membranes (typically absent in prokaryotes) as a result of the need to assemble the photosynthetic apparatus, which facilitates ATP formation.
in light, but without oxygen formation [3]. This growth mode can be implemented in anaerobic photobioreactors where biomass yields of more than 9.2 grams per liter dry weight (gDW/L) have been achieved (unpublished results).

The production of the homologous water-transport membrane protein Aquaporin Z (rAqpZ) from R. sphaeroides was utilized as a model system to develop the screening approach. Expression levels were quantified by monitoring sample fluorescence and Western Blot densitometry (WBD). Initially, monomeric Banana YFP (mBanana) fusion was chosen as a fluorescent tag because antibodies are commercially available and there is no overlap between the fluorescence spectrum of the protein and that of R. sphaeroides. To demonstrate the utility of such correlation for optimizing protein expression, temperature effects were studied as a simple optimization parameter. Ambient and optimal growth temperatures (25°C and 32°C, respectively) for Rhodobacter sphaeroides were studied under anaerobic photoheterotrophic conditions, required to induce protein expression from the puc-promoter used to drive MP expression. Correlations between off-line sampled culture fluorescence and WBD were developed through the use of pure protein standards to demonstrate the feasibility of using fluorescence measurements as the screening method for quantifying membrane protein levels inside growing cells.

A substantially improved signal-to-noise ratio could be achieved by diluting cell suspension in bovine serum albumin (BSA) to provide for refractive index matching and adjust the relative magnitude of light scattering to absorption. Similar trends were observed in MP production per cell at both temperatures, although the productivity was much higher at 32°C due to faster growth. This work identified several opportunities for improving this screening method towards the development of an online monitoring method to track the expression of MPs in vivo.

**Results**

A conversion factor between fluorescence intensity and western-blot densitometry was established for all of the three expression targets using purified proteins. Figure 1 shows the linear relationship between fluorescence intensity and western-blot densitometry for RsAqpZ-mBanana fusion protein. Fluorescence had a much better dynamic range compared to western-blot, which is especially relevant for online monitoring of high-density culture systems.

![Figure 1.](image)

**Figure 1.** a) Correlation between fluorescence intensity and western-blot densitometry, b) Relationship between protein mass and fluorescence intensity
Once we established the correlation between protein mass and fluorescence intensity (Figure 1.b), the extent of the expression throughout the culture growth was possible to monitor direct measurement of the fluorescent intensity with corresponding filter sets for mBanana fusion proteins. We demonstrated determination of the optimum growth temperature for the highest expression titer for each one of the target MPs.

For the homologous RsAqpZ protein, the highest expression titer was reached at 34°C, which was also reported as the optimum growth temperature (Figure 2). AqpZ gene from E.coli was reported to be upregulated in the logarithmic growth phase, which implies the growth-associated expression of this gene in closely related gram-negative bacteria. Human proteins occludin and AQP9 had the highest expression titer at 25°C, which indicates that reduced growth rate promotes the protein expression versus biomass generation.

Although the mBanana fluorescent fusion served as an effective method for correlating membrane protein concentration, these measurements could only be taken offline. The requirement for offline measurement results because variants of GFP (green fluorescent protein) such as mBanana are obligate aerobic fluorescent proteins and require oxygen exposure to fluoresce properly. This presents a significant experimental constraint due to the required anaerobic growth conditions. Maturation involves folding in the presence of oxygen, during which time the fluorescence level per protein increases. Measurements of mBanana-MP fusion maturation within Rhodobacter showed that mBanana required more than 40 hours to mature (20 times its literature value). This long maturation time further precludes the use of this protein for rapid and online monitoring. Ongoing research seeks to overcome this limitation by using a new generation of facultative anaerobic fluorescent proteins based on riboflavin-binding activation.

Conclusions and Future Work

In this study, we demonstrated the utility of fluorescent proteins in monitoring the expression conditions for MP expression in Rhodobacter sphaeroides. The optimum culturing temperature for high expression yields were determined for selected expression targets. In the future, we are planning to expand our expression targets as well as other environmental parameters such growth medium and light intensity for
photoheterotrophic growth and study the effectiveness of using EvoGlow-Pp1 and Evoglow-Bs1 derivatives commercially available from EvoCatal as fusion tags that will hopefully allow correlations with in vivo measurements as well as online productivity monitoring.

References