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OPTIMIZATION OF GROWTH TEMPERATURE CONDITIONS FOR THE
PRODUCTION OF HETEROLOGOUS AND HOMOLOGOUS MEMBRANE PROTEINS IN
ANAEROBIC PHOTOHETEROTROPHIC RHODOBACTER SPHAEROIDES

A Thesis in
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by
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ABSTRACT

We describe the use of fluorescent protein fusions as a method for optimizing and accurately quantifying membrane protein expression in Rhodobacter. Membrane proteins (MP) are a difficult class of proteins to study; however, their medical and biotechnical importance makes them compelling to address the associated challenges. Difficulties of expressing membrane proteins have been reported in number of other protein expression systems such as *Escherichia coli*, yeast and mammalian cell culture. 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, without oxygen formation. The genetic engineering strategy focused on the use of the natural photosynthetic expression machinery to drive expression of the MP of interest in *Rhodobacter sphaeroides*. Anaerobic photoheterotrophic growth can be implemented in anaerobic photobioreactors where it has been previously proven to achieve biomass yields of more than 9.2 grams cells dry weight per liter (gDW/L) have been achieved.

The expression of homologous and heterologous proteins was studied using fluorescent fusion tags and optimized with respect to temperature. The homologous water-transport membrane protein Aquaporin Z (rAqpZ) and heterologous human kidney Aquaporin 9 (human AQP9) and human Ocluddin (human OCLN) were successfully expressed in the Rhodobacter. Expression levels were quantified by monitoring sample fluorescence and Western Blot densitometry (WBD) of Monomeric Banana YFP (mBanana). 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 in vivo.

To demonstrate the utility of such correlation for optimizing protein expression, temperature effects were studied as a simple optimization parameter. Cold, ambient and optimal growth temperatures (16°C, 25°C and 32°C, respectively) for Rhodobacter were studied under anaerobic photoheterotrophic conditions, required to induce protein expression from the puc promoter used to drive MP expression. The expression of both the heterologous and homologous membrane proteins studied was found to be optimum at ambient temperature (25°C). Expression of the homologous and heterologous proteins using mBanana as a reporter were estimated around 3.5 mg-Protein/L-culture and 0.5 mg-Protein/L-culture after 24 hours of culture time.

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 imposed for the expression of membrane proteins in Rhodobacter. Maturation of the fluorescent proteins 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 monomeric literature
value). This long maturation time further precludes the use of this protein for rapid and online monitoring.

Riboflavin-binding fluorescent proteins (RBFP) represent an alternative to the problem of obligate aerobic fluorescent proteins. RBFPs have the ability to absorb and emit light in the absence or presence of oxygen. In this research, pGlow-Pp1 “Evoglow” RBFP (commercially available from EvoCatal) was used as an alternative fusion tag to track the expression of the membrane proteins. The facultative anaerobic RBFP was shown to be an effective measure of concentration of membrane proteins in vivo and “online” under the anaerobic process conditions set for expression. Future work will focus on the use of these tags and correlations, to optimize and understand the expression of membrane proteins online using other Rhodobacter promoter genes and culture conditions.

Statistically significant differences were found between the predicted concentrations using different fluorescent proteins. Underestimation was apparent when using the aerobic mBanana compared to Evoglow. Fluorescent protein stability and sample time was attributed to these differences. Further studies on the practicality and accuracy of Evoglow as a reporter protein for membrane protein expression need to be done as a continuation of this project. Future work should focus on the use of these tags and correlations, to optimize and understand the expression of membrane proteins online using other Rhodobacter gene promoters and culture conditions.
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I would also like to take the time to share this quote that give me motivation to work for the benefit of society:

“(...) Technology isn’t intrinsically good or evil it is how you use it. Like the death ray” – Professor Farnsworth, Futurama.

May we all find our spot in the world of science for the good of others.
Chapter 1

Objectives and Motivation

The study of membrane proteins is one of the new challenges for biotechnological research. In spite of their importance in the medical and life sciences, the structure and function of the vast majority of membrane proteins are not well understood, and only a few membrane proteins have been investigated in detail (Kleinschmidt, 2003; Rabilloud, 2009). Some of the reasons for the lack of knowledge can be attributed to the limited technologies available for expressing membrane proteins in the large quantities required for their study (Kleinschmidt, 2003). A “boom” in the expression and study of membrane proteins has increased exponentially during the 2000’s (Bill, 2011). Industries interested in the production of the membrane proteins have also found the process to be difficult as it uses techniques with low-yields that often are not feasible at a commercial scale. Chemical engineers can provide solutions and insight to this problem by establishing production methods that introduce system feedback in order to increase the production of membrane proteins.

Production of membrane proteins can be viewed as an upstream and downstream chemical process. The upstream operations entail the expression of membrane proteins while the downstream includes their separation and purification. In the early stages of membrane protein production, upstream processes are the most interesting to study. Upstream processes have a vast potential for optimization since they can range from the selection of the organism to the environmental parameters being used for production. Methodologies to accurately predict the levels of protein expression can provide insight
for engineers to maximize membrane protein production and minimize operational cost. Batch time reduction, increasing volumetric productivity, and maximization of titer are amongst some of the benefits of studying the upstream operations. Furthermore, upstream processes have a significant impact on the yield and efficacy of the downstream operations, making it a vital point for study. Downstream purification techniques are also of high importance to the production of membrane proteins. Nonetheless, these techniques require the establishment of efficient upstream processes that will then dictate the hurdles that need to be overcome during the isolation of membrane proteins. For such reasons, we believe that the development of scalable downstream operations will occur in the future.

Techniques to study the production of proteins in vivo and online need to be developed in order to track and predict the expression of proteins in upstream processes. Knowing the titer and volumetric productivity of culture batches online represents a significant advantage when determining the optimum time for harvest of proteins. Tracking protein expression can be achieved using tags such as fluorescent proteins or chemiluminescent reactive antibodies. However, there is no universal method that can be used for tracking the expression of proteins as methods needs to prove effective under the specific process conditions set for production.

Online and in vivo quantification techniques also prove important given that they can be used for optimization of upstream process parameters. Temperature, lighting, gas composition and media selection are among the most commonly optimized parameters in biochemical processes. Optimizing these conditions can greatly improve process yields, minimize operational cost and maximize profits.
The initial goals for this research are:

- Use and establish *Rhodobacter sphaeroides* as a robust organism capable of expressing functional membrane proteins under a wide array of process conditions.
- Develop a method that permits the online and *in vivo* quantification of expressed membrane proteins.
- Study the production of homologous and heterologous membrane proteins in the organism chosen.
- Use the quantification technique to optimize temperature as an example environmental parameter.
- Determine the strength and weaknesses of the technique developed and optimize the methods.

Along the research experiments developments were seen regarding the methods being studied. The study of fluorescent proteins, which were selected as the reporters, became important for the research. Process conditions presented problems with “common” aerobic fluorescent proteins and the studies had to move towards the use of novel proteins. Ultimately, these membrane proteins achieved the goal of this research: provide quantification techniques for membrane proteins.
Chapter 2
Introduction and Background Information

Introduction

The field of biotechnology has greatly expanded over the years as it shows great potential to develop technologies that improve the quality of human life. A greater number of biological molecules are now being studied and produced in large scale bioprocesses in order to satisfy the demand of these products in the medical, food and cosmetic industries. Proteins and antibodies are amongst the group of biological macromolecules that have caught the attention of biotechnology and pharmaceutical companies whose interest rely on technologies that would be more environmentally and medically advantageous. In the class of proteins, membrane proteins (MP) are of increasing interest given their importance in cellular life and the efficiency of medical treatments (Bill, 2011).

Cellular Membrane & Membrane Proteins

More than half of current pharmaceuticals are estimated to target transmembrane proteins (Flower, 1999). This makes membrane proteins an extremely relevant biotechnological product to study in order to improve the efficiency of current drug treatments. Despite their physiological and medical importance, the complexity and
relative low abundance of these proteins in tissues (with human tissue in particular being difficult to acquire) has been a major hurdle to research efforts. However, recent research has focused on the production of biological cell membranes and proteins in more easily accessible microorganisms in order to extract these valuable macromolecules for study.

Membrane proteins (MP) are a class of polypeptides embedded in cell membranes (biomembranes). Both the biomembrane and the MP are vital components in the structure and organization of the elements inside and outside of cellular organisms. Biomembranes, shown in Figure 2-1, are the protective barriers by which the inside substrates are retained and the cell content is stored. Cell membranes are composed of phospholipid molecules whose amphiphillic characteristics allow for existence of both hydrophilic and hydrophobic regions that prevent the passage of water and other molecules. These membranes are mechanically resilient and impermeable to compounds in order to prevent the undesired escape or entrance of these solutes into the cell.

Figure 2-1. Schematic diagram of typical biological cell membrane. (Boumphey, 2009)
The membrane proteins incorporated in these biomembranes are the means by which single-cell organisms interact with their environment and cell-to-cell communication occurs within multi-cellular organisms. Making up to 30% of the entire cell mass, these proteins are amongst the most abundant in the cell given their biological importance (Kleinschmidt, 2003 & Luckey, 2008). Figure 2-2 shows a few examples of membrane proteins along with their functionalities. Membrane proteins have a vast array of functions within the cell; from the joining of biomembranes, to transporting solutes across the membrane and even the translation of the signals in the environment to the inside of the cell (Kleinschmidt, 2003 & Luckey, 2008).

![Figure 2-2](image)

**Figure 2-2.** (A) Schematic diagrams of typical membrane proteins types. (B) Membrane proteins can be both integral (embedded) and peripheral (free floating). (Hedin et al., 2011)
Linking between cells and transport across membranes are considered as two of the most relevant functions of membrane proteins (Luckey, 2008). The transporter proteins are crucial for the maintenance and equilibrium of solute and water balances within the cell, while the linking proteins are integral to the formation of tight junctions between the cells as well tissue (Engel & Gaub, 2008). Amongst the most important proteins able to accomplish the transport and linking function are those of the Aquaporin and Claudin families, respectively (Borgnia, 1999; Feldman, 2005). The relevance of these proteins in the biotechnology field can be appreciated by the fact that the discovery of Aquaporin earned the researchers the 2003 Nobel Prize in Chemistry (Agre, website).

**Aquaporins**

Aquaporins (Figure 2-3) are a family of 10 integral membrane proteins that passively and selectively regulate the flux of water and other small molecules (Verkman & Mitra, 2000). The hourglass shape of the protein selectively allows the transport of one single molecule of water or other molecules at a time (Kumar et al. 2007). Expressed in both eukaryotic (mammals, plants, amphibians, yeast) and prokaryotic (bacteria) organisms, these membrane proteins serve the purpose of regulating the osmotic gradients within the cell (Verkman & Mitra, 2000, Hashido, 2007, Jensen, 2011). Aquaporins are most prominent in the multicellular eukaryotes, given that their large cellular size requires rapid water movement to prevent cell lysis (Soupene, 2002).
Eukaryotic mammalian Aquaporins, namely AQP0 through AQP12, have been reported in a number of important human organ tissues such as the kidney, spleen, liver and brain (Verkman & Mitra, 2000; Elkjaer et al., 2000; Isaksson et al., 2012). This allocation in a wide variety of tissues give Aquaporins another reason to be at the top of the research list in terms of biomedical research applications. One of the most prominent of the eukaryotic Aquaporins is the mammalian AQP9. This highly important member of the Aquaporin family is involved in the transport of water and other small molecules such as glycerol and lactose (Badaut & Regli, 2004). This capability to transport glycerol has deemed the AQP9 an Aquaglyceroporin. AQP9 has been found in organ tissues such as those for the liver and brain, where it regulates important water/glucose gradients across the cell membrane (Badaut & Regli, 2004). Downregulation of the protein has been associated with both neurological and hepatic diseases such as Parkinson’s disease and bile flow dysfunction (Amiry-Moghaddam, 2005; Calamita, et al., 2008). In prokaryotes, Aquaporins are present in bacterial organisms like *Escherichia coli* and *Shigella flexneri*.
as well as archea like *Methanothermobacter marburgensis* (Soupene, 2002; Araya-Secchi, 2011; Lee, 2005). The importance of these Aquaporins is still debated.

The bacterial Aquaporin (AqpZ) is a highly water selective channel that resembles its mammalian counterparts. It is responsible for the adjustment of bacterial organisms to varying tonic conditions (Calamita, 1998). Naturally forming tetramers, Aquaporin Z is a highly robust protein able to withstand voltage, heat, detergents and a wide range of pH conditions (Borgnia, 1999). The high selectivity and stability make Aquaporin Z ideal for an array of biotechnological products; however, competitive economics get in the way of this technology becoming a reality in the near future (Shannon et al., 2008). Laboratory groups such as the Kumar Laboratory at The Pennsylvania State University are trying to use AqpZ in biomimetic membranes for water purification. The group hopes to have functional systems for desalination that would require less energy and be more selective than the current reverse osmosis processes.

*Claudins and Occludin*

Membrane proteins, as mentioned above, not only have transport properties but they also contribute to the tissue and cell-to-cell joining. Cell linking is particularly important in eukaryotic organism as it allows for the formation of functional tissue (Myal et al, 2010). Tissue structures are composed of multiple cellular systems tightly joined together, which then form a barrier (epithelial and/or endothelial). Without cell junctions, the structural integrity of the tissue could never be formed as cells would be
individualized (Cummins, 2012). Amongst the most important cell-to-cell connections are adherens, gap and tight junctions (TJ) - they are shown in Figure 2-4.

**Figure 2-4.** Schematic of cell-cell junctional complexes (Myal et al. 2010).

Other functions aside from tissue formation have been specifically attributed to TJ. Cell differentiation, proliferation and gene expression are some of the functions that are attributed to the development of these junctions (Matter, 2005; Bald, 2009). The down-regulation of TJ has been associated with a wide number of diseases including cancer, stroke and inflammatory bowel disease (Cummins, 2012). Proteins from the Claudins family are the major macromolecules involved in the formation of TJ (Feldan, 2005). Occludin, a integral part of the TJ along with other proteins the Claudin family, was one of the first discovered and characterized proteins in tight junctions.

The role of Occludin is to provide stability to tight junctions and prevent the disruption of the connection (Cummins, 2012). Present in numerous tissues (brain, gastrointestinal tract, eyes), Occludin has been described as a vital protein for proper
tissue formation (Feldan, 2005; Cummins, 2012). The disruption of Occludin has also been connected with a number of diseases. Current research efforts are trying to find ways to prevent and/or reverse Occludin down-regulation through the study of the protein as it may prove beneficial as a therapeutic target.

Membrane Protein Expression

Even though the membrane proteins described above have important biomedical and biotechnical advantages, their study has been difficult. Low concentrations of proteins make the study of several viable Aquaporins (such as mammalian AQP9 and AQP2) and Occludins difficult. Expression of functional membrane proteins around 0.2 mg/L are used as a reference level for comparing different expression systems (Sarramegna et al., 2003; Isaksson et al., 2012). This concentration is orders of magnitude less than would be considered typical for the expression of globular proteins and makes the study of MP difficult and expensive. As an example advances in the study of G-Protein Coupled Receptor (GPCRs) structure, a highly medically relevant set of proteins, required major investments ($600 millions) and decade-long collaborations between the NIH and the European Membrane Protein consortium (E-MeP).

Advancements in heterologous expression of these membrane proteins are viewed as one of the major tools towards overcoming the barriers to their study (Bill, 2011). When considering the organisms to be used for expression of homologous and heterologous proteins, prokaryotes have been preferred over their eukaryotic counterparts (Shuler, 2002). For decades, bacterial systems such as E. coli have been amongst the
most widely used for the expression of proteins given their relatively easy growing conditions and genetic manipulation (Shuler, 2002). The large-scale feasibility for the expression of proteins using bacteria can be seen in cases such as the production of human insulin and angiogenesis proteins in *Escherichia coli* (Goeddel, 1979; Dieckman et al. 2006). However it is important to note that optimization efforts with already available expression systems have provided only minor improvements in overall production of MP (Lian et al. 2008; Andre et al., 2006).

In order to innovate the field of MP expression, new expression systems need to be found. *Rhodobacter sphaeroides* has been suggested as a novel model organism given its potential for producing MP. *R. sphaeroides* has an extensive and inducible intracellular photosynthetic membrane, which allows for large area to volume ratios available for the insertion of membrane proteins (Curtis, 2011; Laible, 2008).

**Expression of Membrane Proteins**

*Rhodobacter sphaeroides* (Figure. 2-5) is a purple non-sulfur, facultative photosynthetic Gram-negative bacteria from the *Proteobacteria Alphaproteobacter* class that naturally produces high levels of intracellular membrane (Jenney, 1993; Choudhary, 2007). Some of *Rhodobacter sphaeroides* advantages as an expression organism for membrane proteins are: (1) a facultative ability to grow under aerobic and anaerobic culture conditions, and (2) the potential to grow under photoheterotrophic (anaerobic) environment. Anaerobic culture conditions are exceptionally interesting for future large-scale production of proteins as they eliminate the limitations of oxygen transport into
solution to promote chemoheterotrophic growth studied in systems using aerobic platforms such as *Escherichia coli* (Rice, 1978 & Losen et al. 2004). The lack of aerobic conditions and the consumption of organic acids as carbon substrates required by *Rhodobacter sphaeroides* reduce the chance of contamination and also make the operational strategy of these reactor systems fairly simple. Moreover, photobioreactor systems studied in the Curtis Laboratory have demonstrated unprecedented biomass yields of 9 gDW/L under anaerobic photoheterotrophic conditions (Curtis B, 2011).

![Figure 2-5. Scanning electron microscopy of *Rhodobacter sphaeroides* cultures. Note the formation of intracellular membrane – circles – within the cell (Donohue, 2002)](image)

In terms of the genetic engineering available for *Rhodobacter sphaeroides*, Argonne National Laboratories has developed and patented a variety of expression systems, described in Chapter 3, for a large library of *E. coli* membrane proteins in *Rhodobacter sphaeroides*. The Curtis Laboratory has studied and developed culture techniques that have optimized the growth of *Rhodobacter sphaeroides*, and the production of membrane proteins in this system (Curtis B, 2011, This work). ANL and Curtis Laboratory yields of these expression systems ranged between 1–20 mg/L and 25–63 mg/L of purified protein, respectively (Laible et al., 2008 & Curtis B, 2011).
Expression Tracking

The growth of *Rhodobacter sphaeroides* and expression of their products (i.e., MP) highly depends on the physicochemical environmental conditions (Shuler, 2002). Even though microorganisms have the ability to grow in a variety of physical, chemical and nutritional conditions, they have specific conditions at which the cells grow in the most efficient manner. These so called “optimal growth conditions” are set to produce a certain outcome in the growth of the microorganism, for example: large biomass or protein yields or fast growth rates (Shuler, 2002). Some of the conditions, such as temperature and culture time, may cause a decrease or increase in the production of the specific products from the cells and vary the outcome of the reaction.

Understanding the expression of these products, MP specifically, has been a difficult task. Determining whether the MP are primary (growth associated) or secondary (growth dissociated) metabolites is amongst the first steps to understand how to produce proteins in large scale operations. This task is not easy as it requires the study of the production during and after growth. Such tracking requires the fusion of the MP to specific reporter tags that do not interfere with the growth of Rhodobacter. These tags should be able to point out the MP using either destructive or non-destructive methods, the latter being the most preferred. Over the past decade, fluorescent proteins have been seen as a great non-destructive reporter that can be used for the study of protein production in biological systems (Tsien, 1998).


**Fluorescent Protein Tags**

Reporter fluorescent protein tags have become essential in the imaging and study of protein expression in biological organisms. Since the discovery of the green and red fluorescent proteins from the *Aequoria victoria* jellyfish and the *Discosoma* mushroom coral, optical microscopy techniques for determining the expression of proteins in vivo have significantly improved (Patterson, 2001; Su, 2005). Heterologous proteins fused with fluorescent proteins have been used to determine expression and location within the cell in both prokaryotic and eukaryotic organisms solely under aerobic conditions (Chalfie et al., 1994; Liu et al., 2001 & Albano et al. 1998). Albano et al. (1998) studied the expression of chloramphenicol acetyl transferase by fusing it with GFP in *E. coli* and optimized the expression by modifying the environmental parameters by tracking the fluorescence.

In order to accommodate the use of different systems, researchers have performed mutagenesis of the jellyfish GFP and the coral DsRed proteins to develop proteins that fluoresce from blue to red colors under a variety of conditions (Tsien, 1998). The use of appropriate fluorescent protein tags can allow for the tracking of the biological product while still maintaining its functionality. The selection of the appropriate fluorescent protein depends on the autofluorescence of the cultures that are going to be used. In Rhodobacter (a purple red bacteria) the presence of carotenoids and bacteriophylls requires the use of specific absorption and emission wavelengths for the fluorescent protein. In order to effectively track the expression of membrane proteins by fluorescence
inside of *Rhodobacter sphaeroides*, the excitation and emission fluorescent tags should be in between the green and orange spectrum (575 nm > \(\lambda\) > 450 nm).
Chapter 3

Methods and Materials

The materials and methodology used for the attainment of the experimental results for this thesis are presented.

Bacterial Cultures

Escherichia coli Top10 and S17 cultures were used for plasmid cloning and transformation steps, respectively. E. coli cultures were maintained on Luria Bertani (LB) media in both liquid and agar forms (appendix Table A.1). Transgenic cultures of E. coli were maintained on 10 µg/mL tetracycline (Sigma-Aldrich). Stock cultures of the transgenic samples were stored in 1:1 volumes of the culture and sterile 50% glycerol solution. The mixture was kept in cryogenic vials at -80°C with minimal thawing cycles.

Argonne National Laboratory (ANL) provided the wild type (WT) and light harvesting antenna knockout (LH knockout) cultures of Rhodobacter sphaeroides 2.4.1 for the expression of membrane proteins. The protocol for the creation LH knockout strain is presented in the literature (Laible et al., 2009). Wild type and non-transgenic cultures of R. sphaeroides were maintained on yeast culture concentrate (YCC) medium in both liquid and agar forms (appendix Table A.2). Transgenic cultures of R. sphaeroides with the pRKPLHT7 plasmid were maintained the same YCC media with 3 µg/mL tetracycline (Sigma-Aldrich). Stock cultures of the transgenic and wild type cultures were also stored in 1:1 volumes of the culture and sterile 50% glycerol solution. The mixture was kept in cryogenic vials at -80°C with minimal thawing-freezing cycles.
**Inoculum Cultures Conditions**

Rhodobacter inoculum cultures were prepared using a sterile plastic loop pressed against the frozen cryogenic stock and then streaked on YCC plates. Cultures were incubated at a temperature of 34°C for a period of two days. Transgenic Rhodobacter cultures were plated on selection of 3 µg/mL of Tetracycline. Once individual colonies were observed, a sterile micropipette was pressed against one colony and placed in a 25mm glass culture tube with 5 mL of YCC liquid medium and the corresponding selection. The cultures were allowed to grow for a period of two days (OD 660 nm corresponding to 1.5-2) and then used to inoculate the media that would be placed in the environmental conditions for the experiment.

**Tetracycline**

Tetracycline in powder and solution form were purchased from Sigma-Aldrich (CAS Number: 64-75-5). Stock solutions were prepared at 15 mg/mL in 95% ethanol. Tetracycline stocks were stored at -20°C, covered with aluminum foil to prevent photoxidation to inactive forms that may generate toxic reactive species (Halling-Sørensen, 2002).

**Proteins and Gene Sequences**

The following proteins were used in these experiments, alongside are their respective DNA sequences.

**Rhodobacter Aquaporin Z – rAqpZ (GenBank: ABA78939.1):** The cDNA of the homologous *Rhodobacter sphaeroides* Aquaporin Z (729 bp) was obtained by PCR
extraction of the genomic sequence. The cDNA was inserted into the MCS of the pRK7 vector without codon optimization. Nucleotide sequence is provided in the appendix Table. B-1.

**Human Occludin** – human OCLN (GenBank: Q16625.1): The cDNA of the heterologous Homo sapiens Occludin (1425 bp) was obtained from The Pennsylvania State University’s Hershey Medical Center. The cDNA was inserted into the MCS of the pRK7 vector without codon optimization. Nucleotide sequence is provided in the appendix Table. B-1.

**Human Aquaporin 9** – human AQP9 (GenBank: BAA24864.1): The cDNA of the heterologous Homo sapiens Aquaporin 9 (891 bp) was obtained from GenBank and was synthesized by GenScript. The cDNA was inserted into the MCS of the pRK7 vector without codon optimization. Nucleotide sequence is provided in the appendix Table. B-1.

**Tobacco Etch Virus Protease Cleavage Site** – TEV: A TEV site was inserted at the C-terminus of three membrane proteins as the linker sequence between the MPs and fluorescent tags: mBanana and Evoglow. The TEV sequence was obtained from GenBank and sent to GenScript for design and creation of the codon optimized version. This linker was selected with the purpose of having a potential cleavage site between the two proteins (Dougherty, 1989); it also provides functional separation between the MPs and the fluorescent proteins. The nucleotide sequence is provided in the appendix Table. B-2.
**Poly-Histidine Tag** – PHT: A PHT was used for the purpose of purification of the protein according to the protocols already established in the literature for membrane proteins (Hengen, P., 1995; Borgnia, M., 1999; Kumar, M. et al., 2007). The nucleotide sequence is provided in the appendix Table. B-2.

**Monomeric Yellow-Orange Fluorescent Protein** – mBanana (GenBank: AAV52167.1): This aerobic fluorescent protein was used as out reporter gene for the production of MPs. Sequence was obtained from GenBank and sent to GenScript for design and creation of the codon optimized version. The mBanana protein was fused to the C-terminus of the TEV site. The nucleotide sequence is provided in the appendix Table. B-3.

**PGlow-Pp1 Evoglow Fluorescent Protein** – Evoglow (GenBank: ADJ00067.1): This riboflavin-binding fluorescence protein (RBFP) was used as the online reporter for the production of MP. Sequence was purchased directly from developer of the protein: EvoCatal in Germany. The nucleotide sequence is provided in the appendix Table. B-3.

**Rhodobacter Transformation: Conjugation**

*Escherichia coli* S17 mediated conjugation of *Rhodobacter sphaeroides* cultures was performed using the protocol developed by Donohue et al. (1991) with modifications by Stephanie Tran (personal correspondence). Mid-log cultures of transformed *E. coli* S17 were added to filter paper (Millipore) and mixed with untransformed Rhodobacter cultures at the maintenance stage. The filter paper with the culture mix was then placed on YCC plates with no selection and allowed to grow for a period of 5 days at an
incubation temperature of 34°C. The filter paper was the collected and soaked and rinsed in 1mL of YCC media for a period of 10 minutes. Of the mix, 100 µL were then plated on MR26+ media with amino acids (appendix Table A-3) plates with 3 µg/mL tetracycline to promote transformed Rhodobacter growth. The visible Rhodobacter colonies where then streaked on fresh YCC plates with 3 µg/mL tetracycline and allow cultures to grow. Individual, transformed, colonies of Rhodobacter were then transferred to liquid YCC media with 3 µg/mL tetracycline, where they were allowed to grow up to an OD of 1 and then used to make cryogenic stocks for future use.

**Photoheterotrophic Growth Conditions and Apparatus**

*Rhodobacter* cultures were grown photoheterotrophically on YCC media under anaerobic conditions. Inoculum cultures were distributed into 25 mL glass tubes filled with fresh YCC media to target an initial optical density of 0.1. Tubes were sealed using a rubber stopper (Bell Labs) with 0.5 mL of air headspace to permit for initial aerobic growth of the culture and prevent culture shock. Tubes were incubated under the specified light conditions and placed in their respective temperatures and gyrated at 8 revolutions/minute using a High-Torque Rotisserie unit (Cole-Palmer). A Model 4629 Force Refrigerated Incubator Shaker (Barnstead/Lab-Line) was used to maintain the temperature in the environment.

**Photoheterotrophic Sampling**

Sampling of the cultures was performed periodically and achieved by exchange of 1:1 volumes of diatomic nitrogen and liquid culture. One mL of Pre-Purified (PP) diatomic nitrogen gas (Air Products) was injected using a 3 mL syringe to maintain
anaerobic culture conditions and be able to extract 1 mL of culture volume without effort. Samples were placed in 1.7 mL Eppendorf tubes and prepared for analysis. The PP nitrogen (Air Products) was run through two 0.2-micrometer filters to insure the sterility of the gas being injected into the samples. Note that all the samples were protected from any other type of lighting during and after the sampling process by being wrapped in reflective aluminum foil. This covering was done to avoid the degradation of tetracycline and the bleaching of the mBanana protein.

**Light Conditions**

Lighting conditions were only applied during photoheterotrophic growth of the Rhodobacter cultures. The cultures were grown under IR LED lighting using 3W 850nm infrared star LEDs purchased from Shenzhen Xuancai Electronic Co. Sixteen IR LEDs were powered by a 1.2A current provided by a 24V DC power supply. The LEDs were mounted to a section of sheet aluminum metal in a two-ring annulus pattern to allow for best light dispersion. The aluminum plate outfitted with LEDs was placed in parallel at an average distance of 8 centimeters with the vertically rotating photoheterotrophic Rhodobacter cultures. Light intensity was measured average 2W/m² during growth. The IR light caused a 2°C temperature increase in the cultures with respect to the incubator temperature.

**Optical Density Measurement**

The cell density was measured using optical density. Samples were placed in 1 cm path length cuvettes and read using a SpectraMax Plus384 Absorbance Microplate Reader spectrophotometer (Molecular Devices), with the absorbance/scattering of the
samples measured at 600 nm for *E. coli* and 660 nm for *Rhodobacter sphaeroides*. The choice of these wavelengths was determined from knowledge of the interference of light-harvesting pigments on the absorbance/scattering. The correlations between optical density, dry weight and cell density for *E. coli* and *Rhodobacter sphaeroides* are available in the literature (Myers et al., 2013) and are provided in Table 3-1.

<table>
<thead>
<tr>
<th>Species</th>
<th>DW/OD&lt;sub&gt;robust&lt;/sub&gt;</th>
<th>CFU/ OD&lt;sub&gt;robust (x10&lt;sup&gt;-8&lt;/sup&gt;)&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>0.396 ± 0.011</td>
<td>7.94 ± 2.0</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>0.453 ± 0.144</td>
<td>367 ± 122</td>
</tr>
</tbody>
</table>

**Table 3-1.** Correlations between the optical density (OD), the dry weight (DW in g/L) and the colony forming units (CFU in cells/mL) of *Rhodobacter sphaeroides* and *Escherichia coli*. (Myers et al., 2013)

The optical density results of the rotisserie samples were targeted below 0.3 A.U. to prevent non-linear effects of the Beer-Lambert law from affecting the correlations already established. Samples were diluted to the target OD using 5% (wt/vol) Albumin, Bovine serum (Sigma). Myers et al. (2013) shows the use of BSA in the attenuation of the cell scattering and enlargement of the light-harvesting pigment responses, which were measured at 875 nm wavelengths.

**Membrane Protein Purification**

To extract the membrane proteins, 1L cultures were pelleted to 10 mL paste and stored at -80C overnight. Pellets were resuspended in 45 mL of lysis buffer (100 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 10 mM imidazole, 1 mM PMSF, 0.1 mg/mL DNase I, 0.02 mg/mL RNase A, 10x PIC, 1 mg/mL Lysozyme, pH 7) and sonicated at 50% duty cycle 5 times in 2.5 minute bursts using a Branson 350 Sonifier cell disruptor. After manual
lysing, the cells were incubated at 34°C for one hour. The cell pellets were spun down at 8,000 rpm for 25 minutes. The remaining supernatant was pelleted out at 47,500 rpm for 1.5 hours. The membrane was then dissolved in 50 mL solubilization buffer (5% β-OG, 100 mM K$_2$HPO$_4$, 10% glycerol, 200 mM NaCl, 20 mM Imidazole, 2mM BME, pH 8) overnight. Any unsolubilized membrane was pelleted out at 47,500 rpm for 1.5 hours, and the supernatant was incubated with 500 µL Co-NTA resin (Thermo Scientific) for two hours. The supernatant was filtered through a 20 mL filter column, leaving the resin bound with protein. The resin was then washed with wash buffer of varying concentrations of imidazole (0/10/20 mM imidazole, 1% β-OG, 100 mM K$_2$HPO$_4$, 200 mM NaCl, 10% glycerol, 2 mM BME pH 7) to be sure the protein eluted would be pure. The protein was eluted with 500 µL of elution buffer (500 mM imidazole, 1% β-OG, 100 mM K$_2$HPO$_4$, 200 mM NaCl, 10% glycerol, 2 mM BME pH 7).

**Membrane Protein Concentration Calculations**

Purified membrane protein concentrations were measured using Bradford colorimetric assay. One mL of a 50:50 salt water-Bradford reagent mixture (IBI Scientific) was mix with 2 µL of the purified membrane protein solution and allowed to react for a period of 5 minutes. The extent of colorimetric change was measured at a wavelength ratio of 360/550, the first being the absorption wavelength of the reacted reagent and the latter of the unreacted. The extent of color change was then correlated to the concentration using a 1 mg/mL Albumin, Bovine serum standard (IBI Scientific) diluted in the 50:50 water-Bradford mixture to create a standard curve.
**Gel Electrophoresis**

Rhodobacter cultures taken at different rotisserie time points were kept frozen at -20°C for the duration of the rotisserie and for one day before being run on the gel electrophoresis. The samples were thawed at room temperature and 200 µL of the culture of each time point were pelleted at 12,000 rpm for 15 minutes. After pelleting, 150 µL of the supernatant was removed and resupplied with 150 µL of lysis buffer (100 mM K$_2$HPO$_4$, 1 mM MgSO$_4$, 10 mM imidazole, 1 mM PMSF, 0.1 mg/mL DNase I, 0.02 mg/mL RNase A, 10x PIC, 1 mg/mL Lysozyme, pH 7) the cells were resuspended and incubated for a period of 15 minutes at room temperature. Cells were then sonicated on a bath sonicator for a period of 15 minutes at 40°C. From the mixture, 5 µL were then combined with 1 µL of 10x reducing agent (Invitrogen), 1 µL 10x Loading Dye (Invitrogen) and 3 µL of sterile ddH$_2$O. Samples were heated at 90°C for 10 minutes. Heated samples were then loaded onto a 10% Novex Tris-Glycine gel (Invitrogen) and ran at a constant 100V for 2 hours. The buffer used was Tris-Glycine SDS Running buffer (Invitrogen) along with 0.5 mL of Antioxidant in the loading chamber of the XCell SureLock Mini-Cell Electrophoresis System (Invitrogen).

**Fluorescence**

The fluorescence of the Rhodobacter cultures was measured using a Tecan Genios Basic apparatus (Tecan). Samples (200 µL/each) were loaded on a transparent 96-well plate (Rainin) covered with reflective aluminum foil to prevent photobleaching of the protein.
For mBanana, cultures were kept in the dark and incubated for 48 hours prior to the reading at 4°C. After the two day incubation period, cultures where placed at 37°C for 1 hour. The fluorescence for mBanana was measured using 535/35 nm (WL/Band Pass) excitation and 590/20 nm emission filters (Omega Optical). Given the poor photostability of the mBanana protein, the number of flashes done by the fluorimeter on the culture was set to 3 and the integration time for the fluorescence emission was set to 20 µs. For the purified protein, the sample was taken directly from the refrigerator at 4°C and incubated for 30 minutes at 37°C. Same fluorimeter settings were used.

For Evoglow, the fluorescence was measured within one hour of sampling. The YCC medium to which the cultures were grown was washed using sterile tap water to avoid background from the Tetracycline (antibiotic emission and absorption spectra resembled Evoglow’s). The culture was brought up to a temperature of 37°C just prior to sampling to insure proper folding of the protein. Fluorescence was measured using 465/10 nm for excitation and 535/35 emission light filters (Omega Optical). Integration and number of flashes of the culture where maintained as for mBanana. For the purified protein, the sample was taken directly from the refrigerator at 4°C and incubated for 30 minutes at 37°C.

**Western Blot (WB)**

The gel with the denatured samples in the electrophoresis step was transferred to a nitrocellulose membrane using the iBlot Blotting system (Invitrogen). Membranes were blocked with TBST buffer supplemented with 5% (wt/vol) non-fat powder milk for a period of 1 hour at ambient temperature. Blocked membranes were then washed with
plain TBST buffer for a period of 30 minutes 3 times at ambient temperature. Subsequently, the membranes were probed at room temperature with the respective Histidine, mBanana or Evoglow antibodies (GenScript, Clontech and EvoCatal respectively) for 1 hour. The membranes were extensively washed with TBST buffer for a period of 30 minutes 3 times at ambient temperature. Membranes were then probed for 1 hour with goat-anti-rabbit conjugated to horseradish peroxidase (GenScript) antibodies that would bind to the primary antibodies. Blots were then washed with TBST and TBS for 40 minutes each. Ultimately, immunobound proteins were reacted using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) and the chemiluminescent response was visualized using the FlourChem II Gel Documentation (Protein Simple) and the image program Alphaview (Protein Simple).
Chapter 4

Membrane Protein Expression in *Rhodobacter sphaeroides*

**Introduction**

The expression and evaluation of membrane protein expression was initiated through a collaboration with Philip Laible and Deborah Hanson at Argonne National Laboratory (ANL). The objective of the project was to establish genetic engineering protocols that would set the groundwork for the expression of homologous and heterologous membrane proteins in Rhodobacter (Curtis, 2011). The genetic engineering strategy is focused on the use of the natural membrane-protein dependent photosynthetic capabilities to drive expression of the MP of interest in *Rhodobacter sphaeroides*.

**Phototrophy of Rhodobacter sphaeroides**

Rhodobacter species of bacteria are capable of converting light energy into chemical energy using a similar process as green plants. Through its photosynthetic capabilities the purple bacteria is able to capture light and use the electrons to build a proton motive force for ATP synthesis (Jackson et al, 2012). This property allows Rhodobacter to grow photoheterotrophically under anaerobic conditions. The photosynthetic apparatus in Rhodobacter is schematically shown in Figure 4-1; it is located in the intracellular and outer membranes of the cell. It consists of a reaction center (RC) and two light harvesting antennas (LH1 and LH2) (Jackson et al, 2012). In this system, the LH complexes allow for the capture and transport of light energy while
the reaction center performs the appropriate photochemistry. LH1 is the largest of the two LH complexes with an RC:LH1 ratio of 1:28 and a light absorption maxima at 875 nm (Hunter, 2009; Conroy, 2000; Bergstrom 1988). LH2 has a variable RC:LH2 ratio larger than LH1, established by the varying biological energy needs of the cell. The LH2 has two absorption maxima at 800 and 850 (Zhao, 2010). The absorption of energy from either LH complex is sufficient to sustain growth, given that in the absence of either one the remaining complex compensates for the deficiency (Hunter, 2005). Under low light conditions the presence of the photosynthetic complexes, especially LH2, increases in order to capture sufficient energy for growth (Hunter, 2005).

![Figure 4-1. Schematic of the arrangement of the photosynthetic apparatus in *Rhodobacter sphaeroides* (Curtis, 2011).](image)

Because of the high-wavelength absorption maxima of *Rhodobacter sphaeroides*, the organism can be grown photoheterotrophically under near-infrared radiation. Curtis (2011) studied the growth of Rhodobacter under near-infrared lighting using 3W Infrared LEDs operating at 850 nm wavelength. The results showed the LEDs were capable of maintaining growth rates similar to those observed under 400W high-intensity discharge lamps (Curtis, 2011). The use of a monochromatic light source proved beneficial when using fluorescent tags to track the expression of MPs, described in Chapter 5.
Given that the photosynthetic proteins described are allocated along the cellular membranes of Rhodobacter, characterizing the genetic pathways behind their production are one of the objectives for the development of techniques for the production of membrane proteins. The promoters inducing the expression of LH1 and LH2 complexes were studied by ANL and the Curtis Laboratory.

**Light Harvesting Complex 2 (LH2) Knockout**

Since the presence of one of the two light harvesting complexes is more than sufficient to sustain growth, ANL studied the removal of one in order to optimize the expression of membrane proteins in the system. LH2 was the chosen complex to remove given its much larger presence within the cell. The knockout of the LH2 complex was done by Laible (2008) at ANL. Figure 4-2, shows the absorption spectra obtained after anaerobic photoheterotrophic growth of wild-type.

![Figure 4-2](image)

**Figure 4-2.** Absorption spectra for photoheterotrophic growth of wild-type *Rhodobacter sphaeroides* cultures (Curtis, 2011).
For the wild type (WT) cultures, as shown in Figure 4-2, the LH2 absorption peaks are clearly available. The peak at 800 nm indicates the presence of the carotenoids of the LH2 peak and the broad peak between 850 and 880 nm shows the overlap between the two absorptions of LH1 and LH2. Figure 4-3 shows the spectra of LH2-knockout strains of *Rhodobacter sphaeroides*.

**Figure 4-3.** Absorption spectra for photoheterotrophic growth LH2 knockout *Rhodobacter sphaeroides* cultures.

In the LH2 knockout strain (Figure 4-3) the lack of the peak at 800 nm can be observed and the presence of a large peak at 875 nm is significantly greater than that of the WT. This larger expression of LH1 seems to be a compensating effect for the LH2 knockout. The removal of the LH2 essentially “emptied” the cellular membrane and creates available space which would lead to improved yields of integrated, functionally-folded MPs. In Chapter 5, the relationship between the overexpression of the LH1 complexes and MP is exploited as a mean of understanding the biological development of the proteins on the cell membrane.
Membrane Protein Genetic Engineering Strategy

Argonne National Labs developed a series of expression plasmids that could be used for the expression of membrane proteins. The most successful of the plasmids for expression was a derivative of the pRK404 vector of the RK2 vector family, shown in Figure 4-4. This vector family contains a \textit{tet}A gene (GenBank: AY204475.1) regulated by a tetracycline repressor protein (TetR) that gives the cultures resistance to tetracycline (Ditta et al., 1985; Pokkuluri et al., 2002; Scott, 2003). The multiple cloning site (MCS) of the pRK404 vector derivative, namely the pRKP-T vector, is shown on Figure 4-5. A poly-histadine tag was added downstream of the MCS to allow for the purification of MPs using chromatography (Laible et al., 2008).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{vector_sequences.png}
\caption{Vector NTI images for the vector sequences used in this work. (A) pRK404 vector (Scott 2003; Laible, 2008). (B) pRKPLTH7 vector designed by ANL for the expression of membrane proteins in \textit{Rhodobacter sphaeroides} (Laible, 2008).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{mcs_design.png}
\caption{Multiple cloning site design in the pRK404 vector derivative used for MP expression.}
\end{figure}
As part of the relationship to the phototrophy of *Rhodobacter sphaeroides*, the production of MP was chosen to be driven by native promoters used in the expression of the photosynthetic apparatus. Native *puf* and *puc* promoters where studied for the expression of membrane proteins (Laible et al., 2008). The *puf* promoter drives the expression of RC and LH1 complexes under semi-anaerobic heterotrophic conditions while the *puc* promoter induces the expression of LH2 under anaerobic photo-heterotrophic conditions (Laible et al., 2008). ANL and Curtis Laboratory have studied the use of both promoters along with the differences in MP expression. The work found the photoheterotrophic *puc* promoter to be the more productive of the two systems, which in turns concurs with the expression of the LH2 complexes in the wild-type Rhodobacter. From these results, the Curtis Laboratory developed the membrane protein expression in Rhodobacter using the pRK vector driven by the *puc* promoter, namely the pRKPLHT7 vector. Figure 4-6 schematically shows the genetic engineering strategy being used in this work.

![Figure 4-6](image)

*Figure 4-6*. Schematic of the genetic engineering strategy being used for the expression of MP using native promoters.
Cloning of Membrane Proteins

The initial cloning work was performed by Mustafa Erbakan (personal correspondence) and verified by the author along the course of the experiments. The cloning of single membrane protein sequences into the pRKPLHT7 vector was done through insertion via the SpeI and BglII restriction sites. The MP nucleotide sequences provided for Rhodobacter Aquaporin Z, Human Aquaporin 9 and Human Occludin were inserted between the SpeI and BglII restriction sites downstream from the puc promoter and upstream from the poly-histidine tag (PHT). The result is schematically shown in Figure 4-7.

![Figure 4-7. Schematic of the cloning done to the MCS in order to insert the MP sequences.](image)

Successful insertion of the membrane protein sequences was verified by sequencing and also by double digestion of the cloned pRKPLHT7 vectors, shown in Figure 4-8. MP vectors were then conjugated into *Rhodobacter sphaeroides* LH2 knockout cultures previously prepared and grown under anaerobic photoheterotrophic conditions to induce protein expression. The expression of the membrane proteins was verified by Mustafa Erbakan (personal correspondence) using Western Blot analysis of purified MPs from Rhodobacter.
Once the expression of the MPs using the pRKPLHT7 vector was confirmed, the fusion of these proteins was developed in order to develop MP production tracking techniques.

**Fusion of Membrane Proteins to Fluorescent Protein Reporter**

The MCS of the pRKPLHT7 vector was modified in order to do easy fusion tag drop-ins. In this work, done by Mustafa Erbakan (personal correspondence), an AgeI restriction site was inserted between the SpeI and BglII sites to allow the insertion of MPs and fluorescent tags separately (Figure 4-9A). The DNA sequence of the mBanana protein fused with Tobacco Etch Virus cleaving sites were cloned into the vector in between the AgeI and BglII sites. The TEV recognition site was added in this sequence in order to provide a post-translational cleaving site that would allow for the separation of
the MP from the fluorescent protein to study the effects of the tag on the performance of the protein. Lastly, the MP DNA sequences for rAqpZ, human AQP9 and human OCLN were inserted as previously discussed to yield the MCS site schematically represented in Figure 4-9B.

![Figure 4-9. Schematic of the cloning done to the MCS in order to insert the MP fused with Fluorescent Protein Tag sequences. (A) Insertion of AgeI site for drop ins. (B) Final MCS product with fluorescent tags.](image)

LH2 knockout Rhodobacter cultures were transformed and grown anaerobically under photoheterotrophic conditions. The cloning was verified by sequencing and the expression was confirmed by fluorescence readings of the purified protein and Western Blotting. The verification work was all done by Mustafa Erbakan (personal correspondence). Once all the vectors have been developed and all the sequences were confirmed the expression of membrane proteins was undertaken.

In this research, monomeric Banana (mBanana; 544 nm max. excitation, 553 nm max. emission) was chosen as the fluorescent tag for the membrane proteins given that the fluorescence spectrum (Figure 4-10) does not overlap with that of *Rhodobacter sphaeroides* (Tsien, 1998; Shaner et al., 2004).
The final molecular weights and DNA sizes of the MP along with its mBanana (mBa) fusion partner is provided below in table 4-1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>rAqpZ</th>
<th>hAqp9</th>
<th>hOcc</th>
<th>TEV</th>
<th>mBanana</th>
<th>PHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Size (bp)</td>
<td>729</td>
<td>891</td>
<td>1425</td>
<td>27</td>
<td>708</td>
<td>27</td>
</tr>
<tr>
<td>Molecular Weight (kDa)</td>
<td>28</td>
<td>31</td>
<td>59</td>
<td>0.9</td>
<td>27</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 4-1. Weights and DNA sizes for MP and their fusion partners in the pRKPLHT7 vector.
Chapter 5

Correlations Between Optical Density, Fluorescence and Western Blotting

Introduction

To achieve high throughput screening of a wide range of conditions rapidly without the need to perform expensive and labor-intensive analysis, it is necessary to have accurate in vivo screening. Multiple biotechnological and pharmaceutical companies have been trying to achieve rapid screening methods in order to optimize their batch productions (Drew et al., 2005; Backmark, 2013). This chapter describes the series of experiments performed in order to find relationships between the different techniques used for the data analysis of the growth experiments to track and study the production of membrane proteins in vivo and online. The objective is to find a non-destructive and easy method for the accurate determination of the protein concentration within the cell at any point in time during culture growth. Offline measurements were carried out on sampled cells with rAqpZ fused with mBanana protein to determine the correlation between fluorescence, Western Blot densitometry (WBD) and optical densities at 660 and 875 nm. The cells were sampled at different times during the growth experiment according to the procedures established in the methods and materials section.
Results and Correlations

*Rhodobacter sphaeroides* samples were grown on a rotisserie unit under photoheterotrophic conditions at 25°C (incubator temperature), and samples were taken periodically and analyzed accordingly. The fluorescence, densitometry and optical density results were then compiled and plotted against the time the cultures were grown under infrared light, referred to as LED time. Optical density (OD) measurements were done by diluting the sampled cell culture in 5% Albumin, Bovine serum solution and taking both the 660 and 875 nm absorption of the sample. Use of the 5% BSA solution was done to improve the OD signal to noise ratio of the culture (Appendix C). The results for the correlation experiment are presented in Figure 5-1.

![Graphs showing correlation results](image)

**Figure 5-1.** Plot of the different measurements performed during the correlation experiments.
The exponential trend of the three data sets suggests that there is a direct relationship between each of the measured parameters. Three experimental replicates at the same temperature (25°C) were performed, and the fluorescence, optical density and densitometry point were plotted against each other to observe the relationships between the parameters at any given point in time. Figure 5-2 shows the correlation of the Western blot densitometry and fluorescence.

![Figure 5-2](image)

**Figure 5-2.** Plot of the fluorescence versus densitometry showing linear relation.

The results of this experiment confirm the anticipated correlation between the protein measuring techniques: Fluorescence and Western Blot densitometry (WBD). The trend line was fitted to zero to reflect the logic that without protein, there is neither fluorescence nor WBD for that sample. If fluorescence and WBD did not yield a straight line, then this would have indicated that there was saturation of the densitometry measurements or a reading outside of the dynamic range of the fluorescent reading. The correlation between the optical density and the protein measurement techniques is shown in Figure 5-3, below.
Figure 5-3. Relationship between the optical density of the culture and the protein measuring techniques. (A) Fluorescence against optical density. (B) Western blot densitometry against optical density against.

These trends indicate that the production of membrane proteins was directly related to the growth of the cells, which made the protein a growth-associated product that is made during the exponential phase. The linear relationship between the optical density and protein readings indicate that the yield of protein per cell was constant. The values of the slopes for the relationships appear to hold only at the temperature for which the correlations have been developed, given that there are changes in the biomass to protein production energy and nutrient consumption.
Discussion

The use of arbitrary units in Figures 5-2 and 5-3 does not give much of an idea of the accuracy of this approach to evaluate the concentration of protein in the cell. In order to ameliorate such situation, the arbitrary units of both fluorescence and western blotting were standardized to basic units using calibration standards. Purified protein of known concentration was used to develop the calibration curves. The fluorescent spectroscopy calibration for mBanana protein, used in Chapter 6 to quantify expression, is shown in Figure 5-4.

![Fluorescence arbitrary units with respect to mass of fluorescent protein. Calculations are shown in Appendix D.](image)

A similar calibration curve constructed using several western blots was also developed to relate the WBD to protein mass. The results are shown in Figure 5-5.
Figure 5.5. Relationship and verification of validity of the two selective protein quantification methods – WB and fluorescence. Standards were used to convert the arbitrary units of both pieces of equipment.

Given this relationship, the results of both the Western blot and fluorescent spectroscopy measurements correlated 1:1 to each other within the range of protein level in the cells and protein loading of the Western blot. This also demonstrated the accuracy of the relationship between the two-protein quantification methods and also made a point regarding the redundancy of the measurements. The deviation in the top right corner point of Figure 5-5 may suggest a saturation of the densitometry reading at high concentrations of MP. This then points that fluorescence is a slightly more accurate reading than WBD at higher concentrations since the fluorimeter does not saturate.

One important advantage that stemmed from using mBanana fluorescent protein is that calibrations between the fluorescent, WBD and protein concentration should be maintained regardless of the MP fusion partner. This occurs because mBanana is the only protein that should fluoresce under the excitation and emission of the fluorimeter and also
be the only protein that binds to the specific primary antibody in WB. Overall, with this techniques and correlations, the concentration of any protein can be determined through simple scaling of the fluorescence and WBD of the mBanana to which it is attached.

The expression and production of membrane proteins could furthermore be studied using the relationship between the expression of the light harvesting protein LH1 (Myers et al. 2013). As mentioned in Chapter 3, the photosynthetic protein promoters are used in the expression of the MP of interest. The *puf* heterotrophic promoter drives the expression of the RC and the LH1 complexes during anaerobic growth. This expression is similar to that of the *puc* photoheterotrophic promoter used to drive the expression of MP. Even though not under the same promoters, it should be possible to track the extent of membrane protein being expressed under anaerobic conditions using the absorbance of the LH1 protein being expressed (measured at 875nm). The ratio of the absorbance at the two wavelengths (875/660) is used to observe the expression of LH1 complexes per cell. Results for this ratio as a function of time are presented in Figure 5-6.

![Figure 5-6](image.png)

**Figure 5-6.** Plot of the 875/660 wavelength ratio as a function of time. Presents relationship between LH1 complex expression and culture optical density. LH2 Knockout has no pRKPLTH7 vector.
The results show the increasing expression of the LH1 complex during cell
growth and the asymptotic behavior of the absorption of the complex at high density.
Differences in LH1 expression can be observed between the LH2 knockout cultures
without pRKPLTH7 vector and with that expressing rAqpZ mBanana fusion MP. The
LH2 knockout appears to have consistently higher 875/660 ratios than the MP expressing
strain. This could be explained but the ability of the LH2 knockout strain to express and
allocate a larger number of LH1 antennas than the transformed culture, given that this
one is occupying membrane space with the rAqpZ-mBanana MP. These results point to
the fact that this relationship can be used to qualitatively follow the extent of MP
expression in the system.
Chapter 6

Use of Fluorescence Screening to Optimize Temperature Conditions for Membrane Protein Production

Introduction

In this study, the effects of temperature will be studied with the intention to determine whether or not a lower temperature than the ‘optimal’ can improve the production of membrane proteins in *R. sphaeroides*. Previous studies on the effects of protein production done in *E. coli* (Jazini, 1996; Andersen 2013) have shown conflicting results with respect to choosing temperatures above or below the growth optimum. Jazini et al. (1996) demonstrated that the expression of secreted globular proteins was improved when temperatures were 9°C lower than the optimum for growth (37°C) but did not improve further with lower temperatures. Andersen et al. (2013) studied the effects of temperature oscillations in the production of proteins and found that oscillating temperatures around the optimum by ±2°C was beneficial to the production of soluble proteins in *E. coli*. The production of functional membrane proteins appeared to benefit from membrane synthesis; therefore, it might be particularly sensitive to temperature.

Lower temperatures have been hypothesized to be beneficial for the production of membrane proteins, given the fact that lower temperatures retard the growth of microbial cultures. This reduction in growth rate then allows for more time and energy to be used for creation of proteins rather than biomass. This premise is particularly interesting for photoheterotrophic growth, where energy from light is used to produce ATP, thereby
potentially decoupling growth rate and energy availability. *Rhodobacter sphaeroides* grows under a variety of temperatures (4–35°C), however temperatures between 28 and 34°C under both photoheterotrophic and chemotrophic conditions have been considered optimum (Boone, 2001; Curtis, 2011).

**Results**

**Effect of Temperature on Growth Rate**

Cultures of LH2 knockout, empty pRKPLTH7 vector, rAqpZ, human AQP9 and human OCLN pRKPLTH7 vectors and their respective fusions with mBanana protein were grown at three temperatures: 18°C, 25°C and 34°C. The objective is to determine the effects that these temperatures have on the growth rate and protein production. Using optical measurements, the culture density was tracked as a function of time during the exponential growth phase (OD < 6). An exponential curve was fitted to obtain values for the growth rate. The growth rates as a function of temperature are shown in Figure 6-1, below.

![Figure 6-1](image)

**Figure 6-1.** Effect of temperature on the growth rate of *Rhodobacter sphaeroides* cultures.
When considering the meaning of a growth rate and the effects temperature has on them, it is somewhat easier to think of the time it takes for the culture to double. In the spirit of visually being able to see the effects of temperature, the doubling time of the cultures based on their growth rate is presented in Figure 6-2, below.

**Figure 6-2.** Effect of temperature on the doubling time of *Rhodobacter sphaeroides* cultures.

First and foremost, this work evaluated the effect of temperature on the growth rate. Understanding how much temperature affects the growth rate of cultures is an important parameter that can aid in the upstream process and reactor designs. Growth rates were found to have a positive correlation with temperature on all cultures. The LH2 knockout and empty pRKPLHT7 vector cultures are used as the control cultures for comparison with those expressing MP. The rate of growth of the control cultures was determined to be the same with 95% confidence at the two higher temperatures, which in turn indicates that tetracycline exposure and resistance does not affect the growth of the cultures. At low temperature it was noted that the doubling time of the LH2 knockout
culture was reduced (meaning faster growth), pointing to the fact that negative antibiotic effects might occur at lower temperatures. The growth rate of the MP expressing cultures was determined to be the same as the MP-mBanana expressing partner cultures with an 85% confidence at all temperatures.

Results shown on Figures 6-1 and 6-2 indicate under anaerobic photoheterotrophic conditions, *Rhodobacter sphaeroides* cultures grew preferentially at the higher temperature with the maximum (of the three temperatures) around 34°C. The growth of MP expressing cultures was slower than the controls regardless of temperature, with human AQP9 being the slowest. This result points to the fact that the load of the MP and MP-mBanana production is a source for the growth rate reduction. However, it is important to note that the length of the MP appeared to have minimal effect (<15%) on the growth rate of the cultures. Figure 6-3 shows these results.

<table>
<thead>
<tr>
<th>Protein</th>
<th>rAqpZ</th>
<th>rAqp9</th>
<th>rAqpZ mBa</th>
<th>hOcc</th>
<th>hAqp9 mBa</th>
<th>hOcc mBa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular wt (kDa)</td>
<td>28</td>
<td>31</td>
<td>55</td>
<td>59</td>
<td>59</td>
<td>87</td>
</tr>
</tbody>
</table>

**Figure 6-3.** Effects of membrane protein molecular weight on the doubling time of *Rhodobacter sphaeroides*. 
Homologous Rhodobacter AqpZ Expression

After establishing the protocols for tracking the production of membrane proteins (Chapter 5), MP expression was followed using fluorescence of the cultures. The production of each individual MP was studied at the different temperatures. Results of this tracking were plotted as content (mg MP/gDW cells) and titer (mg MP/L culture) versus time in order to gauge the expression as a function of time. Culture titer is both a content and culture density dependent quantity, equal to the product of these two values. The results for the expression of the homologous Rhodobacter Aquaporin Z are shown in Figure 6-4.

![Graph A](image1)

![Graph B](image2)

**Figure 6-4.** In vivo rAqpZ protein expression in *Rhodobacter sphaeroides* at different temperatures. Determined using cultures expressing rAqpZ-mBanana fusion protein. (A) Graph of protein content as a function of time. (B) Graph of titer as a function of time.
Results from Figure 6-4 indicate that expression of the homologous rAqpZ MP in *Rhodobacter sphaeroides* is dependent on both temperature and time. Cell protein content is demonstrated to be optimum at 25°C. Figure 6-4A shows the protein content per cell at 25°C increases as a function of time until it reaches a maximum around 2.3 mg MP/gDW cells. Protein content at 34°C also increases as a function of time, however it levels off at 2.0 mg MP/g DW cells. MP content at 18°C is shown to increase up to 1.2 MP/g DW cells in the first 15 hours of LED time and then drop and remain somewhat constant around 1 mg MP/gDW cells for the rest of the growth.

The amount of protein in the cells appears to have been more influenced by the time allowed for the culture to grow and adapt to the change between chemoheterotrophic and photoheterotrophic growth. Given that membrane protein production was driven by the *puc* promoter that would normally express the LHII peripheral light-harvesting complex, the gradual increase towards the end of the culture could reflect the response to light limitation as the cell density increased.

Protein production per liter of culture was greatest at the highest temperature given the fact that there was more culture and cells available to produce the protein. The production of rAqpZ MP at the end of each batch run was calculated to be 8.9, 5.1 and 0.8 mg MP/L at 34, 25, and 16°C respectively. These values for unpurified culture protein concentration are around the same order of magnitude as the concentrations obtained for purified MP (Erbakan, personal correspondence).
Expression of the heterologous Human Aqp9 protein was studied under a variety of temperatures and tracked the same way as the homologous rAqpZ. Cultures expressing this protein were found to be the slowest growing of all the transgenic lines, which in turn affected the titer. Results for the expression of the heterologous Human Aqp9 are shown below in Figure 6-5.

**Figure 6-5.** *In vivo* human AQP9 protein expression in *Rhodobacter sphaeroides* at different temperatures. Determined using cultures expressing human AQP9-mBanana fusion protein. (A) Graph of protein content as a function of time. (B) Graph of titer as a function of time.
Results from Figure 6 indicate that the expression of the heterologous human AQP9 MP is lower than the expression of the homologous rAqpZ. It also shows that the titer is dependent on both temperature and time while the content appears to be unaffected by either parameter. Cell protein content was below 0.25 mg MP/gDW cells and was relatively similar for all temperatures. Figure 6-5A shows the optimum cell protein content of 0.23 mg MP/gDW cells expressing at 25°C. The second best cell content expressing temperature was 18°C with a maximum of 0.15 mg MP/gDW cells. Cell protein content at 34°C was not constant during the run and even decreased significantly from 0.16 to 0.07 MP/gDW cells after 10 hours of culture time.

Protein production per liter of culture was found to be greater at the intermediate temperature since both the culture density and content were beneficial for the productivity. The production of human AQP9 MP at the end of each batch run was calculated to be 0.43, 0.79 and 0.08 mg MP/L at 34, 25 and 18°C, respectively. These values for unpurified culture protein concentration are around same order of magnitude as the concentrations obtained for purified MP (Erbakan, personal correspondence).

**Heterologous Human Occludin Expression**

Expression of the heterologous Human Occludin protein was studied under the same variety of temperatures as the human AQP9 and rAqpZ cases. Expressing cultures of the human OCLN proteins were found to be nearly on par in growth rate with those expressing homologous rAqpZ. Results for the expression of the heterologous Human Occludin are shown below in Figure 6-6.
Figure 6-6. *In vivo* human OCLN protein expression in *Rhodobacter sphaeroides* at different temperatures. Determined using cultures expressing human OCLN-mBanana fusion protein. (A) Graph of protein content as a function of time. (B) Graph of titer as a function of time.

Figure 6-6 indicates that the expression of the heterologous human OCLN resembles that of human AQP9. Both titer and content appear to be dependent on both temperature and time. Cell protein content was below 0.2 mg MP/gDW cells and with small differences between all temperatures. The optimum cell protein content of 0.19 mg MP/gDW cells was achieved at 25°C. The second best cell content expressing temperature was 18°C with a maximum of 0.14 mg MP/gDW cells. Cell protein content
at 34°C constantly remained below that of the lower temperature averaging a low 0.05 mg MP/gDW.

Protein production per liter of culture was once again found to be greater at the intermediate temperature of 25°C. Production of human OCLN MP at the end of each batch run was calculated to be 0.31, 0.57 and 0.05 mg MP/L at 34, 25 and 18°C, respectively.

Membrane Protein Productivity Analysis

In order to establish which of the three temperature conditions was the most beneficial for the production of the MP; volumetric productivity calculations were done using time and culture density points. The final predicted concentrations of the MP proteins were calculated at the 1 day and 1 gDW/L-culture time points. Using these two points it is possible view the optimum temperature for MP expression using both a “specific” and volumetric productivity of unpurified protein. The results for the expression of MP after 1 day of culture are shown in Figure 6-7, below.
Homologous membrane protein expression was found to be most productive on a daily basis at 34°C. A 24-hour batch rAqpZ concentration of 5.3 mg/L was the highest achieved followed by 2.8 mg/L at 25°C. Heterologous protein production per liter after 24 hours was consistently greater at 25°C yielding 0.53 mg/L for human AQP9 and 0.44 mg/L for human OCLN. All MP productivities at 18°C were found to be at least one order of magnitude below that at the higher temperature ones. Results based on culture density (1 gDW/L) are shown below in Figure 6-7.
Figure 6-8. Protein productivity (mg MP/L-culture) when culture reaches 1 gDW/L of MP at different temperatures.

The homologous membrane protein expression was found once again to be the most productive when compared to the heterologous expression. Expression of rAqpZ at a culture density of 1 gDw/L was found to be optimum at 25°C with a titer of 1.85 mg/L. Heterologous protein production at 1 gDW/L was, once again, consistently greater at 25°C yielding 0.27 mg/L for human AQP9 and 0.44 mg/L for human OCLN. MP productivities at 18°C and 34°C were found to be relatively the same at culture densities of 1 gDW/L.

In conclusion, the optimum temperature for the production of MP, based on the daily and specific productivities, is 25°C. The use of *Rhodobacter sphaeroides* as a model system for the expression of membrane proteins demonstrates promising qualities as it has the capability of expressing both heterologous and homologous proteins. Results show MP concentrations above 0.2 mg/L for heterologous and 1.0 mg/L for homologous
protein production standards (Sarramegna et al., 2003). These results indicate that once fully optimized, *Rhodobacter sphaeroides* may actually prove to be a viable platform for the production of membrane proteins at large scales in the future.
Chapter 7

Use of mBanana Fluorescent Protein Under Anaerobic Conditions

*Introduction*

One reoccurring problem that delayed the off-line measurement of the fluorescence was the long maturation time of the mBanana fluorescent protein. Full maturation of pure mBanana protein under aerobic conditions takes 1 hour at a temperature of 37°C (Tsien, 1998). However, even though efforts were made to accommodate the recently sampled cells at these temperatures under fully aerobic conditions, the cells did not show their maximum fluorescence. The working hypothesis for the delay of the fluorescence on behalf of the protein has to do with the fact that the mBanana remains in the intracellular space, where oxygen diffuses more slowly. An experiment to observe the maturation and fluorescence of the mBanana as a function of time was performed in order to find the time of maturation within the cell.

In order to avoid error by having the cultures grow in the 96-well plate during the fluorescence spectroscopy measurement, samples were kept at a low temperature of 4°C to retard their growth as much as possible while also exposing them to aerobic conditions. A study on the maturation time and determination of the peak fluorescence was performed by having photoheterotrophically grown cultures sampled and exposed to oxygen for a period of 50 hours while periodically taking fluorescence readings of the protein. The results are shown in Figure 7-1.
Figure 7-1. Determination of the maturation time of the mBanana protein within the cells through the fluorescence emission.

The results indicate that the maturation time (peak fluorescence) of the fused mBanana protein within the cell laid between 40 and 50 hours. The incremental fluorescence points to the fact that the protein progressively (and slowly) gets oxygenated and appropriately folds within the cell. It was observed that the fluorescence of the protein went down slightly between the 45 and the 50 hours reading. This decrease in the fluorescence is due to the photochemical bleaching that occurs during the fluorescence measurement when the protein was excited with multiple flashes of light. Particularly high energy light exposure can irreversibly bleach the mBanana fluorescent protein and prevent it from fluorescing once again. This poor photostability of mBanana is considered to be a negative trait as it can cause the fluorescent correlations to vary significantly and yields lower values due to the bleaching of protein.
Both photobleaching and the obligate aerobic conditions required for maturation are a major hurdle when using traditional fluorescent proteins. The errors associated with the high photobleaching (poor photostability) of mBanana can be minimized by using more photostable proteins such as eGFP and mKOK. However, the use of more photostable proteins will still not solve the problem of the obligate aerobic conditions required for photochemistry. Further study of the environmental parameters in the production of membrane proteins under photoheterotrophic (anaerobic) conditions using fluorescent protein reporters will require the use of newly synthesized anaerobic fluorescent proteins. mBanana has been shown to not be the best fluorescent protein available and we have proven this to be a fact in our research with the time delays this protein caused (Tsien, 2005).

In order to get around this fact, we decided to use riboflavin-binding proteins, which have the ability to fluoresce under the anaerobic photoheterotrophic conditions in Rhodobacter as shown by Drepper et al. (2007). Even though the brightness and photostability of these proteins are about same as mBanana, it is expected that this will bring higher accuracy to the fluorescence readings and also improve the correlations between fluorescence, optical density and Western blotting. These fluorescent proteins can then also be used in large scale operations and serve as online monitoring reporters that would track the expression of the MP in situ without the need to take analyze samples offline.
Chapter 8

Evoglow Genetic Engineering and Fusion Partner Expression

Introduction

Although the fluorescent fusion proteins served as a simple and effective method for determining the protein concentration in vivo, the use of obligate aerobic fluorescent proteins was a significant experimental inconvenience. For such reason, this work looked to use new fluorescent fusion reporters such as the riboflavin-binding fluorescent proteins (RBFP) developed by Drepper et al. (2007). Riboflavin-binding protein fluorescence occurs without the need for oxygen since their photochemistry is based on the chemiluminescence reaction of flavonoids. The full chemical reactions behind this photochemistry are still not entirely known, however their expression and fluorescence have been shown under both aerobic and anaerobic conditions (Drepper et al. 2007).

Figure 8-1. Fluorescence of RBFP ‘Evoglow’ under aerobic and anerobic conditions (Drepper et al. 2007).
**Cloning**

In this research, dimeric pP1-pGlow ‘Evoglow’ (pP1-Evoglow; 450 nm max. excitation, 495 nm max. emission) developed by EvoCatal was chosen as the RBFP tag for the membrane proteins. This protein was selected based on the recommendations of the provider and also because its fluorescent spectrum (Figure 8-2) does not overlap with that of *Rhodobacter sphaeroides* (Drepper et al., 2007). The DNA sequence for this protein was cloned using the pRKPLTH7 vectors modified for FP drop-ins mentioned in Chapter 4.

**Overlapping PCR**

One important problem that arose during this cloning was the presence of a restriction site (AgeI) used in the MCS of the pRKPLTH7 vector in the middle of the Evoglow protein (Figure 8-2).

![Figure 8-2. Problem presented by the presence of an AgeI restriction site in the DNA sequence of Evoglow.](image)

In order to solve this problem, a single nucleotide in the Evoglow sequence was changed to remove the restriction site. When considering doing the sequence change, the nucleotide replacement must not cause protein translational differences. The nucleotide change was performed using overlapping PCR. For the overlapping PCR, four different
primers were used and two PCR reactions were performed. The process is shown in Figure 8-3, below.

![Diagram](image)

**Figure 8-3.** Overlapping PCR technique for single nucleotide exchange in Evoglow.

As shown in Figure 8-3, the product of the overlapping PCR was a restriction free Evoglow sequence. After obtaining and purifying this product the ‘corrected’ Evoglow sequence was verified using sequencing. Once the DNA sequence of the protein was verified, the protein was fused in the pRKPLHT7 vector with the heterologous and homologous MP proteins in this study. The final result for the cloning is shown in Figure 8-4.

![Diagram](image)

**Figure 8-4.** MCS of the pRKPLHT7 containing MP-RBFP fusion partners.
The insertion and fusion of the Evoglow sequence into the pRKPLHT7 vector with MP was proven by PCR of the sequence. The results are shown in Figure 8-5.

**Figure 8-5.** MCS of the pRKPLHT7 containing MP-RBFP fusion partners. (1) NEB 1K bp Ladder; (2) Negative Control, (3) rAqpZ-Evoglow, (4) human OCLN-Evoglow; (5) human AQP9-Evoglow; (6) NEB 100 bp Ladder.

The expression and fluorescence of Evoglow as a MP fusion partner was confirmed for all proteins. The RBFP was purified as a fusion with rAqpZ and the correlation between protein fluorescence and mass was developed (Figure 8-5).

**Figure 8-5.** Correlation between fluorescence (arbitrary units) and the mass of fluorescent protein. Calculations are shown in Appendix D.
Fluorescence Correlations

Once all correlations were developed and the fluorescence of Evoglow was confirmed, the use of Evoglow as a MP production-tracking partner was studied. Transformed Rhodobacter cultures with pRKPLTH7-MP-Evoglow and mBanana were grown for a period of 24 hours. The fluorescence was measured at the end of the run and their final protein concentration was calculated using the correlations previously developed. The mBanana fluorescence was measured 48 hours after sampling while the Evoglow fluorescence was measured immediately. The results are plotted in Figure 8-6.

![Graph](image)

**Figure 8-6.** Comparison between the expression predicted by the Evoglow and mBanana reporter proteins. Cultures were grown for a period of 24 hours.

Even though grown at the same conditions, the expression of MP calculated from the use of Evoglow was consistently higher than that of mBanana for all MP studied. This difference in the correlation could be attributed to a variety of reasons. The protein fluorescence and stability is dependent on the time samples are removed from their
expression environment. Since the fluorescent reading for Evoglow was taken more promptly than the mBanana one, it can be assumed that the fluorescence and stability of Evoglow is better than that of mBanana. However, performing fluorescent protein studies similar to the one shown in Figure 5-5 are required in order to prove this assumption. This analysis will be done in the future work along with a tracking of the MP using Evoglow as the fluorescent reporter.
Chapter 9

Conclusions

The production of homologous (rAqpZ) and heterologous (human AQP9 and human OCLN) membrane proteins in Rhodobacter sphaeroides was studied using fluorescent proteins. Expression of membrane proteins in Rhodobacter was confirmed by purification and reporter techniques. The relationship between optical density, fluorescence and Western blot densitometry was studied using the mBanana fluorescent protein as both the Western Blot and fluorescent tag. The correlation between the Western Blot and fluorescent analytical techniques was proven to be accurate with respect to one another. Fluorescence was then determined to be a simpler and faster technique to determine the concentration of protein in vivo.

Production of the membrane protein during growth was tracked under a variety of temperature conditions in order to find the optimum. Protein concentration in vivo was calculated using the correlations developed. The production of the homologous Rhodobacter Aquaporin Z was found to be volumetrically (mg MP/L) optimal at 34°C and specifically (mg MP/gDW Cell) at 25°C. The expression of heterologous human membrane proteins Aquaporin 9 and Occludin was found to be volumetrically and specifically optimal at 25°C. In vivo culture protein concentrations, calculated through fluorescence methods, were a function of time using the obligate fluorescent protein mBanana.

The specific and volumetric productivities calculated for the proteins were studied using mBanana at two different batch times: 24 hours and when cultures reached 1
gDW/L. The titer for the expression of rAqpZ at ambient temperature was estimated to be 3.5 mg-Protein/L-culture at 24 hours and 2.2 mg-Protein/L-culture at culture density of 1 gDw/L. Titer of the heterologous proteins at 24 hours and 1 gDw/L were found to be 0.53 and 0.27 mg-Protein/L-culture for hAqp9 and 0.44 and 0.40 mg-Protein/L-culture for human OCLN.

The use of the obligate aerobic fluorescent mBanana was found to have major weaknesses in terms of its fluorescence under anaerobic Rhodobacter growth conditions. The efficiency and expression of the novel riboflavin-binding facultative anaerobic fluorescent protein ‘Evoglow’ was studied in order to find the best reporter protein under the anaerobic growth conditions. Evoglow was shown to be expressed and fluoresce under anaerobic conditions while fused to the membrane proteins. Significant differences were found between the predicted concentrations using fluorescent proteins. Underestimation was apparent when using the aerobic mBanana compared to Evoglow. Fluorescent protein stability and sample time was attributed to these differences. Further studies on the practicality and accuracy of Evoglow as a reporter protein for membrane protein expression need to be done as a continuation of this project.
Chapter 10

Ongoing and Future Work

Experiments to establish Evoglow as the fluorescent protein reporter are currently being undertaken. The goal is to develop correlations between fluorescence and Western Blot densitometry similar to those created for mBanana. With these correlations, the protein expression tracking of the various membrane proteins will be done at one temperature to prove the efficiency of the system developed.

After the development of methods for tracking the expression of membrane proteins have been developed, new methods of expression and production can be now studied. Optimization of environmental and genetic engineering promoters can also be done with the methods presented in this thesis. Light and nutrients are the next environmental conditions to be optimized. The comparison between the productivity of the photoheterotrophic \textit{puc} and heterotrophic \textit{puf} promoter will be the next condition to be optimized for the expression of membrane proteins.

Once all environmental and genetic engineering techniques are optimized, this work will head towards the large-scale production of homologous and heterologous membrane proteins in Rhodobacter. The large-scale expression will be done using the state of the art ($20 million) pilot plant facility at Penn State.

Aside from Rhodobacter Aquaporin Z, two other proteins have been chosen for further study: the tight junction protein Occludin and the human kidney aquaporin (Aqp9). The study and optimization of these proteins will signify a major advance in the
field of medical membrane protein production. Techniques and optimized conditions for Rhodobacter growth can also be used for the production of biofuels as the research for new sources of fuels shifts towards the use of microbial organisms.
Appendix A

Recipes for Culture Mediums

This section provides the media recipes for the solutions used during these experiments. Note that all of these solutions were made fresh from the components presented. YCC media medium was used for general maintenance and experimentation with *Rhodobacter sphaeroides* and *Escherichia coli* cultures; LB medium was used for growth of *E. coli* cultures; MR26+ ammonium succinate defined medium was used for cloning steps with Rhodobacter.

Below the recipe for LB media is presented:

<table>
<thead>
<tr>
<th>Media Component</th>
<th>[Stock]</th>
<th>Added to 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td></td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td></td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>5 mL</td>
</tr>
</tbody>
</table>

The solution is brought to 1L with ddH$_2$O and pH-adjusted to 7.5.
The medium is autoclaved for 25 minutes at 121C, cooled, and stored aseptically.

For solid 5 g/L of Agar was added before autoclaving.

Table A-1. Luria Bertaini (LB) media recipe.

Below the recipe for YCC media is presented:

<table>
<thead>
<tr>
<th>Media Component</th>
<th>[Stock]</th>
<th>Added to 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td></td>
<td>5 g</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td></td>
<td>6 g</td>
</tr>
<tr>
<td>YCC Base Concentrate</td>
<td></td>
<td>5 mL</td>
</tr>
<tr>
<td>Na$_2$EDTA·2 H$_2$O</td>
<td></td>
<td>11.82 g/L</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td></td>
<td>0.04 g/L</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td></td>
<td>0.03 g/L</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$_7$O$_2$·4H$_2$O</td>
<td></td>
<td>0.02 g/L</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td></td>
<td>40.12 g/L</td>
</tr>
</tbody>
</table>
FeSO$_4$·7H$_2$O  0.75 g/L
H$_3$BO$_3$  0.0125 g/L
Ca(NO$_3$)$_2$·4H$_2$O  6.9 g/L

The solution is brought to 1L with ddH$_2$O and pH-adjusted to 7.2.
The medium is autoclaved for 25 minutes at 121°C, cooled, and stored aseptically.

For solid 5 g/L of Agar was added before autoclaving

Table A-2. Yeast Culture Concentrate media recipe.

Below the recipe for MR26+ media is presented:

<table>
<thead>
<tr>
<th>Media Component</th>
<th>[Stock]</th>
<th>Added to 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Succinate</td>
<td></td>
<td>2.36 g</td>
</tr>
<tr>
<td>MR26 Phosphates</td>
<td></td>
<td>20 mL</td>
</tr>
<tr>
<td>K$_2$HPO$_4$ (dibasic)</td>
<td></td>
<td>115 g/L</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (monobasic)</td>
<td></td>
<td>44.9 g/L</td>
</tr>
<tr>
<td>MR26 Micronutrients</td>
<td></td>
<td>1 mL</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td></td>
<td>10.9 g/L</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td></td>
<td>1.3 g/L</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td></td>
<td>0.392 g/L</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td></td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$·4H$_2$O</td>
<td></td>
<td>0.186 g/L</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td></td>
<td>0.114 g/L</td>
</tr>
<tr>
<td>Fe-EDTA Solution</td>
<td></td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Fe-EDTA·2H$_2$O</td>
<td></td>
<td>4 g/L</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>0.234 g</td>
</tr>
</tbody>
</table>

The solution is brought to 1L with ddH$_2$O.
The medium is autoclaved for 25 minutes at 121°C and allowed to cool.
The following solutions are then added aseptically, reducing precipitation:

<table>
<thead>
<tr>
<th>Media Component</th>
<th>[Stock]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium Solution</td>
<td>1.205mL</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td></td>
</tr>
<tr>
<td>Calcium Solution</td>
<td>0.45mL</td>
</tr>
<tr>
<td>CaCl$_2$·2 H$_2$O</td>
<td>150 g/L</td>
</tr>
<tr>
<td>Vitamin Stock</td>
<td>1mL</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>3 g/L</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>3 g/L</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>6 g/L</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.12 g/L</td>
</tr>
</tbody>
</table>

Table A-3. MR26+ media recipe (Argonne National Labs, personal correspondence).
Appendix B

Nucleotide Sequences of Proteins Researched

Below the nucleotide sequences are provided for the proteins used in these experiments. All nucleotide sequences are provided in the FASTA NCBI format.

The nucleotide sequences of the membrane proteins are provided below:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Code</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodobacter sphaeroides</em> Aquaporin Z</td>
<td>rAqpZ</td>
<td>ATGACCAAGAAGCTCCTCCTCGCCGAGCTCCTCAGCCTTCATCCTCCTCG</td>
</tr>
</tbody>
</table>
### Homo sapiens Occludin human OCLN

| ATGGAAGACGAAATCTGCACTTCTACAAATGGACCAGCCCCCCCGG |
| GCCTCATCCGCATCTCTCAGCATCGTCATATGGTGGTTCGAGATCGGACAGCGGCTACG |
| GCACCAGCCCTGCCGTCGCTGAGCACCCTGGCCTGGGACCGCGGCTACG |
| GCACCAGCCTGCTCGGCGGCTCGGTGGGCTACCCGTACGGCGGCAG |
| GCCTTCGCTGCACTGGACGCAGCGTTCATGCTGACATCGTCTCTTCACCATCATCTTTCG |

### Table B-1. Nucleotide sequences for the membrane proteins.

The nucleotide sequences of the linker between the membrane protein and the fluorescent tag is provided below. Furthermore the tag used for separation is provided below:

| Tabacco Etch Virus | TEV | GAGAACCTCTACTTCCAGGGC |
| Poly-Histadine Tag | PHT | CACCACCAACCCACACCAAC |

### Table B-2. Nucleotide sequences for the linkers and separation tags.
The nucleotide sequences of the fluorescent proteins used in this thesis are presented below:

<table>
<thead>
<tr>
<th>CloneTech Yellow-Orange DsRed Variant FP</th>
<th>mBanana</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGGTGAGCAAGGGCGAGGAATACATGGGCACGTCATC</td>
<td></td>
</tr>
<tr>
<td>AAGGAGTTTATCGCGCTTACAGTGAGCTGGAGGCTCC</td>
<td></td>
</tr>
<tr>
<td>GTGAAACCGGACGATTTGCGAGATCGAGGCGAGGCA</td>
<td></td>
</tr>
<tr>
<td>GGGGCCGCCCTACAGGAGCCACCCAGACCGCAAGGCT</td>
<td></td>
</tr>
<tr>
<td>GGTGACCAAGGTGCGCCCTGGCGCTGGGACATC</td>
<td></td>
</tr>
<tr>
<td>CTGTCCCTTCAGTTCTGCTACGCTTGAGCTACGTTG</td>
<td></td>
</tr>
<tr>
<td>AAGCACCCACTTGTTATCCCGACACTTCAAGCTGCT</td>
<td></td>
</tr>
<tr>
<td>TCCCGAGGGCGCCAGTGATCGAGCAGGATGACTTCC</td>
<td></td>
</tr>
<tr>
<td>CGACCAACTTCCCTCCCGACGCCCCTGATCGAGGAAG</td>
<td></td>
</tr>
<tr>
<td>AGAAGACGCACTGAGTGAGGCCTGAGCGGATGACC</td>
<td></td>
</tr>
<tr>
<td>CCGAGCACTCCCGACGCCCCTGAGGCGAGATCGAGG</td>
<td></td>
</tr>
<tr>
<td>CTGAAAGCTGAAAGCGGCGGCGCCTACAGCGAGGAG</td>
<td></td>
</tr>
<tr>
<td>GAGGCCCTATACAGCCACGAGGAAGGCGAGTAGG</td>
<td></td>
</tr>
<tr>
<td>GCTGAAGGAGGGCGGCACTACAGCGGAGGAGGAGG</td>
<td></td>
</tr>
<tr>
<td>GCTGGAGGCTGAAGTGGCGGAACTGCAGGGCAGG</td>
<td></td>
</tr>
<tr>
<td>GCTGAAAGGCGGCACTACAGCGGAGGAGGAGGAGG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EvoCatal pGlow-Pp1 Evoglow</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGATCAACGCAACACTCCCTGGAAGTATGCTGAAAC</td>
</tr>
<tr>
<td>TCCAAACTGCAATCGTCGAGGACGAGGACGGAAT</td>
</tr>
<tr>
<td>GAGAGCATCTTATCTACGCTCAACCAGCCTGAGCC</td>
</tr>
<tr>
<td>TGACCCGCTACTGAGCGCCAGATTTCTCTATACGAGGC</td>
</tr>
<tr>
<td>CCGTTTTTCTGACAGGCGAGGCTACGACGGAGCGAG</td>
</tr>
<tr>
<td>CGCAATATAGGGTCGAGGACGAGTGAGGAGAGGCC</td>
</tr>
<tr>
<td>GTTCGTAAGTCTGCTCTACGCTACGCTACGCTACG</td>
</tr>
<tr>
<td>GGGGACCCAGTGAGCTACATCGGAGCTACGAGGAG</td>
</tr>
<tr>
<td>GCTGAGAGCCTAGATGAGGCGGAAATGCTGAGGAG</td>
</tr>
<tr>
<td>GCTGAGAGCCTAGATGAGGCGGAAATGCTGAGGAG</td>
</tr>
</tbody>
</table>

**Table B-3.** Nucleotide sequences for the fluorescent protein tags.
Appendix C

Optical Density Signal to Noise Ratio Reduction Using BSA

As described in Chapter 5, the use of optical density measurements can significantly simplify the measurement of culture density and protein expression. Myers et al. (2013) describe the use of different dilution mediums in order to improve the accuracy of these measurements by minimizing the background “noise” of the readings. Light measurements in culture measure the level of scattering of the light, not absorption. This means that culture readings at specific wavelengths are chosen to measure how much light is being diffracted off the photosensor by the cells that are in solution. Selected wavelengths (600 nm for E. coli, 550 nm for algae and 660 nm for Rhodobacter) should minimize the amount of background that can be given by absorbing factors such as colored compounds and cell wall refractive index differences.

Matching the refractive index between the cells and the dilution medium is one way to reduce the background noise in optical density readings. Myers et al. (2013) extensively studied the use of BSA to reduce signal to noise ratio of the samples and improve variability of the samples. Figure C-1 shows the minimization of the variability between the 875/660 nm ratio of Rhodobacter cultures grown photoheterotrophically when they are diluted using 30% BSA.
Figure C-1. Effect of using 30% BSA solution as the solvent to make dilutions for readings. Samples at OD 7 were diluted to the read OD 660nm values on the x-axis.

Dilutions using BSA show a consistent pattern of between the 875/660 ratio while the water diluted samples are scattered and do not show much consistency. It was noted that large concentrations of BSA also had a negative effect on the overall cell density measurement. Since BSA “camouflages” differences between the cells and the medium they are diluted in, after using a concentration above 20% BSA the OD measurements were off by an average 15% from the undiluted OD measurement. Study of the BSA concentration pointed to 5% BSA as the best option for both signal to noise ratio reduction and also for accurately measuring the cell density (<1% difference).
Appendix D

Correlations of Arbitrary Units Between Different Fluorescent Proteins

The development of correlations between fluorescent arbitrary units (A.U) and protein mass is required in order to use fluorescence as a mean of tracking protein expressions in cells. Adding a specific mass of the pure fluorescent protein (FP) and measuring its fluorescence in arbitrary units develop these correlations. The mass of protein is added using a concentrated solution of the purified fluorescent protein and adding a specific volume. However, in some cases it is difficult to isolate the single fluorescent protein so the fusion products (MP–FP) are used for the development of fluorescence/FP mass correlations. In this thesis the fluorescence per mass of mBanana and Evoglow were developed using the rAqpZ–FP fusion partners. The correlations of the fluorescence/FP mass of rAqpZ–FP are shown in Figure D-1.

Figure D-1. Fluorescence arbitrary units correlations with MP-FP fusion mass correlations. (A) For rAqpZ-mBanana. (B) For rAqpZ-Evoglow.

As these correlations stand, they cannot be used with MP proteins other than the fusion rAqpZ-FP. Mathematical ratios and calculations are required in order to be able to
these correlations on the other heterologous MP (human AQP9 and human OCLN). The first step in making these correlations universal for all proteins is to determine the concentration of the fluorescent protein and make the correlation of fluorescent/mass of pure FP. The calculations can be done using molecular weight and mole relationships between the fusion and individual proteins. Since the molar concentration of the fusion protein is 1:1 with respect to the individual proteins the calculation is fairly simple. The step by step calculations are shown below in Equations D-1 through D-3.

\[
\frac{[\text{FusP}]}{M_{\text{FusP}}} = \frac{\text{Moles FuP}}{mL \text{ culture}} \quad \text{Equation D-1}
\]

\[\text{Moles FusP} = \text{Moles FP} \quad \text{Equation D-2}\]

\[
[\text{FP}] = \frac{\text{Moles FP}}{mL \text{ culture}} \times M_{\text{FP}} \quad \text{Equation D-3}
\]

**Table D-1.** Equations for the scaling of fluorescence between systems that use MP-fluorescent protein fusions to correlate the value of fluorescent arbitrary units with FP mass.

Where FusP is the abbreviation for the fusion protein and FP for fluorescent protein. Using the equations above, it is possible to convert the correlations made using rAqpZ-FP fusions to fluorescence per mass of pure FP. The graphs for these correlations are shown in Figure D-2.
Figure D-2. Fluorescence arbitrary units correlations with MP-FP fusion mass correlations. (A) For mBanana. (B) For Evoglow.

Once the correlations between pure (individual) fluorescent proteins and the arbitrary units has been calculated, it is simple to relate the numbers to MP. By applying the equations in Table D-1 and knowing the molecular weight of all proteins it is possible to establish all fluorescent correlations. The results for all of the individual proteins are shown on Figure D-3. Differences between the slopes is given by the size of the proteins and the fluorescence per cell.
Figure D-3. Fluorescence arbitrary units correlations with pure MP mass correlations. (A) For mBanana fused MP. (B) For Evoglow fused MP.
References


Jazini, Mohammadhadi. "Effects of Temperature Shifts and Oscillations on Recombinant Protein Production Expressed in Escherichia Coli." *Bioprocess Biosyst Eng*(2013)


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EDUCATION

Doctor of Philosophy degree in Chemical and Biochemical Engineering, May 2017
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Thesis: TBD

Master of Science degree in Chemical Engineering, August 2013
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Thesis: “Optimization of Membrane Protein Expression in Rhodobacter sphaeroides”

Bachelor of Science degree in Chemical Engineering with Honors, May 2013
The Pennsylvania State University, University Park, PA
Schreyer Honors College Scholar

High School Diploma with a specialization in Science, July 2008
Unidad Educativa Colegio Emil Friedman, Caracas, Venezuela.
Summa Cum Laude and Valedictorian

HONORS AND AWARDS

• Engineering College Career Services Student of the Year Nominee 2011 – 2012
• Craig Millar Award for Student Leadership and Service Award 2011 – 2012
• Larry Duda Undergraduate Student Research Award in Chemical Engineering Fall 2011
• Chemical Engineering Honor Society (ΩXE) Student of the Year Award 2011 – 2012
• Vollmer-Kleckner Scholarship 2010 – 2012
• Chevron Engineering Scholarship 2010 – 2011
• Excelsior Prize for Academic Excellence Fall 2007

ASSOCIATION MEMBERSHIPS

• Chemical Engineering Honor Society, ΩXE Fall 2011 – Present
• American Institute of Chemical Engineers Fall 2009 – Present
• Society of Hispanic Professional Engineers Fall 2008 – Present
• Tau Beta Pi, Engineering Honor Society Fall 2011 – Present

PROFESSIONAL EXPERIENCE

Albemarle Corporation, Technology Resources (R&D) Intern – Baton Rouge, LA Summer 2012
• Formulated a computational model for determining thermophysical properties for compounds.
• Improved thermochemical calculations for parameters of fertilizer and catalyst reactions.
• Performed competitive analysis research of lithium extraction processes in Latin America.
Albemarle Corporation, DCS Operations Intern – Pasadena, TX  
Summer 2011
  • Created computer logic and programs for interlocks of valves, pumps, reactors and motors.
  • Rectified programming errors to improve performance of safety interlocks and plant equipment.
  • Designed computerized process flow diagram graphics for on-line plant control systems.

RESEARCH EXPERIENCE

Penn State Chemical Engineering, Research Assistant – University Park, PA  
Research advisor: Dr. Wayne Curtis and Dr. Manish Kumar  
Fall 2012 – Present
  • Using GM *R. sphaeroides* with fluorescent-tagged membrane proteins to study protein expression.
  • Optimizing environmentally changeable parameters to improve batch and protein yields.
  • Performing photoheterotrophic pilot plant bioreactor studies for membrane protein production.

Penn State Chemical Engineering, Research Assistant – University Park, PA  
Research advisor: Dr. Wayne Curtis  
2009 – 2012
  • Prepared viral vectors for the insertion of GUS gene into hydrocarbon producing algae (*B. braunii*).
  • Adapted freshwater algal cultures to high salt levels using a shock-conditioning experiment.
  • Decontaminated extra-cellular hydrocarbon producing algae cultures using antibiotic treatments.
  • Trained incoming research collaborators and students on laboratory techniques and procedures.

ACADEMIC EXPERIENCE

Penn State Chemical Engineering, Teaching Intern – University Park, PA  
Spring 2013
  • Aided perform experiments in Senior Undergraduate Laboratory course (ChE 480).
  • Prepared fluid flow, heat exchanger, process control and reaction kinetics equipments.
  • Instructed students on SOPs for safety and equipment and graded pre and post laboratory questionnaires.

Penn State Chemical Engineering, Teaching Intern – University Park, PA  
Spring 2012
  • Assisted in the instruction of the Introductory Biomolecular Engineering course (ChE 340).
  • Conducted (4) lectures on genetic engineering, pharmaceutical development and growth kinetics.

Penn State Chemical Engineering, Teaching Intern – University Park, PA  
Spring 2012
  • Supported the instruction of the Mathematical Modeling course (ChE 360).
  • Held review sessions and how to lessons for the computer program: Mathematica®.

Penn State Chemical Engineering, Grading Assistant – University Park, PA  
Spring 2011
  • Graded the first level Thermodynamics and Energy Balances course (ChE 220).
  • Corrected homework and special assignments for both honors and regular sections.

Penn State Chemistry Department, Instrument Teaching Assistant – University Park, PA  
Fall 2010
  • Assisted undergraduate students in the use of Nuclear Magnetic Resonance and Infrared apparatus.
  • Performed 150+ NMR, IR and Gas Chromatography on different organic compounds.

Penn State Mathematics Department, Grader – University Park, PA  
Fall 2009
  • Graded first level calculus (MATH 140) under the supervision of assistant professor.
  • Prepared homework assignments, quizzes and reported on the performance of the class.

Penn State Chemistry Department, Laboratory Assistant – University Park, PA  
Spring 2009
  • Volunteered over 50 hours at the chemistry department’s laboratory stockroom.
  • Enforced safe laboratory practices, disposal protocols and attended to student chemical requests.
PUBLICATIONS

Penn State Chemical Engineering, Graduate Research Assistant – University Park, PA


PRESENTATIONS

DOW Sustainability Challenge 2012


American Institute of Chemical Engineers 2010 – 2012


SKILLS

- Computer programming: Mathematica, Honeywell DCS, Visual Basic and Stella.
- Techniques: Axenic culturing, gel electrophoresis, cloning, Western Blot and vector design.
- Instruments: NMR (60 and 400 Hz), Infrared, GC-MS, UV spectroscopy, fluorimeter and HPLC.

EXTRACURRICULAR ACTIVITIES

Professional Association of Diving Instructors, Member – Caracas, Venezuela Spring 2004 – Present

- Logged 50+ recreational dives and three specialty dives.
- Completed The American Red Cross First Aid and CPR courses.
- Achieved the Open Water, Advanced Open Water, Rescue and Specialties diver certifications.