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Development and Characterization of Oxygen Sensitive Microbial Biosensors

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Abstract

Microbial biosensors are useful in detecting suboptimal conditions in a bioreactor. This detection can help in development of improved mixing methods to reduce the occurrence of these conditions. In this research, the activities of 3 promoters known to have activity under anaerobic conditions, the *gadB* promoter, an FNR protein activated promoter, and the *pfl* promoter, were tested for activity under anaerobic conditions by connecting them to a green fluorescent protein (GFP) gene. Using two types of media, Luria-Bertani (LB) and Wilms-Reuss media, the activity of these promoters under aerobic and anaerobic conditions were tested. Results indicate that while the FNR activated promoter and the *pfl* promoter show weak performance as an indicator of anaerobic conditions, the *gadB* promoter exhibited a twofold increase in GFP expression under anaerobic conditions as opposed to aerobic conditions.

Acknowledgments

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Introduction

Fermentation broadly refers to the growth of microorganisms on a growth medium with the goal of producing a specific chemical product. These desired chemical and biological products can range from biofuels to antibodies, and on a small-scale, can more easily be produced in ideal conditions. However, during scale-up to large-scale fermentation, long mixing times and non-homogenous conditions can present difficulties in producing the desired product. Sub-optimal conditions can be detrimental to product formation, as it may lead to reduced biomass and the formation of undesirable byproducts. Thus, developing microbial biosensors that can monitor the oxygen concentration of a fermentation bioreactor are helpful in maintaining optimal conditions for microorganisms that may be in the bioreactor. Eventually, these microbial biosensors be used to improve fermentation through changes in process conditions or by changes in the cell itself. The pyruvate formate-lyase (*pfl*) system, glutamate decarboxylase (*gadB*) promoter, and an FNR protein activated promoter that was previous tested in the cyanobacterium *Synochocystis* sp. PCC 6803¹ were used to test the viability of using them as biosensors. These promoters, whose activities have been found to be turned on under anaerobic conditions, were connected to a green fluorescent protein (GFP) gene as a reporter and then characterized using aerobic and anaerobic conditions. The transcription factors associated with these promoters are the FNR protein and the ArcAB transcriptional regulator. The FNR protein contains an iron-sulfur cluster that is normally oxidized under aerobic conditions; in the absence of oxygen, this protein can dimerize, and in this state is able to bind to DNA and induce transcription⁷. In addition, the ArcAB transcriptional regulator system is composed of ArcA and ArcB; In response to an absence of oxygen, ArcB, a histidine kinase, phosphorylates ArcA, a transcriptional repressor⁶.

Materials and Methods

Plasmid Construction and Cell Transformation

For the *pfl* promoter, colony polymerase chain reaction (PCR) was performed to amplify the promoter using primers that also contained homology with the pKVS-45 plasmid backbone and GFP. PCR mixture was prepared using NEB OneTaq DNA polymerase. Gel electrophoresis was performed on this product to verify the correct product size and then extracted using the Monarch DNA Gel Extraction Kit from NEB. Afterwards, this PCR product was used in overlap extension PCR to connect the insert to the whole GFP gene, again verifying correct DNA size and extracting the DNA from the gel. Finally, using circular polymerase extension cloning (CPEC)², a whole plasmid was formed connecting the pKVS backbone, *pfl* promoter, and GFP gene. To transform cells with this plasmid, chemically competent DH5 α *E. coli* cells were first prepared using SOB broth and stored in a -80 °C freezer; testing of these plasmids was carried out in both DH5 α and MG1655 strains. 100 μ L of competent cells were thawed and then 5 μ L of the CPEC product were added to the cells and allowed to incubate for 30 minutes. The cells were then heat shocked for 30 seconds at 42 °C. 0.4 mL of SOC broth was then added to the cells and incubated for an hour in a shaker at 37 °C. These cells were plated on a Luria-Bertani

(LB) and ampicillin (Amp) plate and left to grow at 37 °C. A colony was later picked and a 15% glycerol freezer stock was made.

The FNR activated promoter was constructed using PCR with a forward primer containing part of the promoter sequence and homology to the pKVS backbone. The reverse primer contained homology with the GFP gene and the GFP gene. A first round of PCR was done to amplify the GFP gene from the pTRC-GFP plasmid. A second PCR was done using a forward primer containing homology with the pKVS backbone and the remainder of the FNR activated promoter. Finally, a CPEC reaction was performed using the pKVS backbone and this PCR product to create a plasmid containing the FNR activated promoter and the GFP gene. This plasmid was transformed into chemically competent cells using the method describe previously and grown on an LB/Amp plate. A colony was picked and a 15% glycerol freezer stock was made.

The *gadB* promoter was cloned during a previous student's work.

Fluorescence Experiments

For each fluorescent experiment, 3 mL of LB starter cultures were first grown for every plasmid to be tested, along with a negative control of the strain with no plasmid, and a positive control of the strain with pTRC-GFP. Ampicillin was added to culture tubes with plasmids to ensure plasmid retention. These cultures were allowed to grow overnight in a 37 °C shaking incubator, and 20 mL of LB medium in a flask were inoculated with 200 µL of starter culture. Each plasmid was tested in duplicate. After 2 hours of growth, a 1.5 mL sample was taken as the initial data point, and half of the volume in each flask was placed in a Hungate tube with a rubber stopper and a screw-on cap. The head space of these tubes were purged by flowing nitrogen gas into the tube for 30 seconds with a syringe needle with another one as an outlet. The tubes and flasks were grown in the shake incubator at 37 °C and samples were taken periodically. These samples were centrifuged in 1.7 mL microfuge tubes for 2 minutes at 13,000 rpm. The supernatant was discarded and the cells were resuspended in 3 mL of 0.9% NaCl solution.

Experiments were also run using Wilms-Reuss medium³, which did not require centrifugation and resuspension. Experiments in this medium were done using the strain MG1655.

Fluorescence was measured using the Fluorolog-3 and DataMax Data Collection software. Data was collected using an excitation wavelength of 490 nm and an emission wavelength of 510 nm. In addition, the slit width was set at 5 nm. Optical density (OD) was measured using the Cary 500 Spectrophotometer at an absorption wavelength of 600 nm. Data was normalized by dividing fluorescence by the OD.

Results

The level of fluorescence in each plasmid indicates that there is GFP being produced, but the difference between aerobic and anaerobic conditions tended to fluctuate, and varied between different promoters and also between strains and medium. DH5 α was not grown in Wilms-Reuss medium because its growth rate in that medium was very slow.

FNR

Before any anaerobiosis, the FNR promoter already shows a very high level of fluorescence. Experiments on this promoter show that the performance of this promoter in LB medium tends to be inconsistent, being more fluorescent under anaerobic conditions only in some samples (Figure 1). However, using the Wilms-Reuss medium resulted in consistent data (Figure 2), showing a higher fluorescence under aerobic conditions. Differences in media indicate that there may be some interaction between the components of these media and the binding sites of the promoters, resulting in more or less gene expression. Previous work done in the cyanobacteria *Synochocystis* sp. PCC 6803 indicates a promoter sensitivity to oxygen for the FNR activated promoter, up to around an 8-fold increase in mRNA concentration, suggesting higher transcription levels, under anaerobic conditions¹. Work done by Moser also indicates higher transcription levels under anaerobic conditions for promoters activated by the FNR promoter⁷.

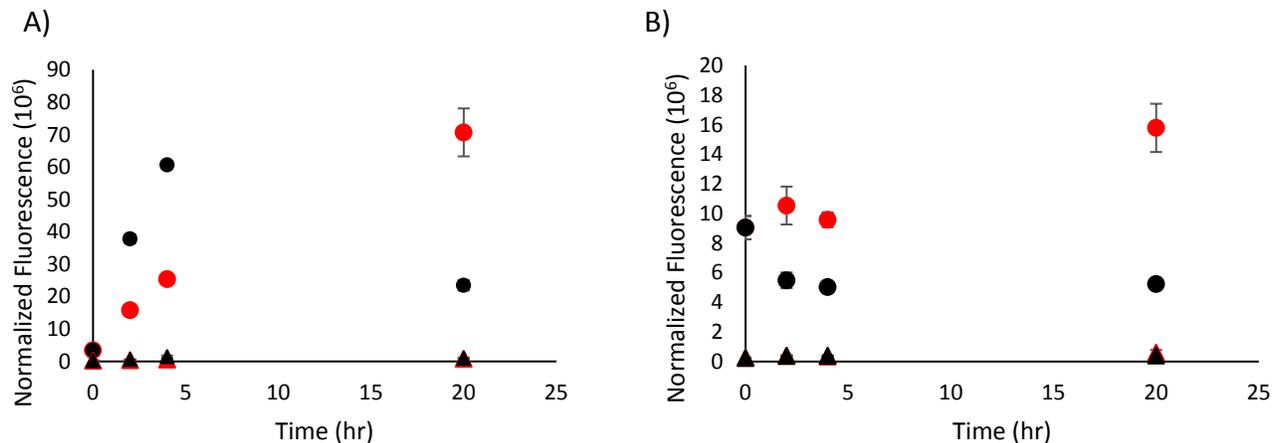


Figure 1. Red indicates aerobic conditions and black indicates anaerobic conditions. The circles represent the FNR plasmid and the triangles are the negative control. Two experiments were run in LB medium, one with DH5 α (A) and the other with MG1655 (B). These results suggest that the FNR activated promoter is more fluorescent under aerobic conditions when in MG1655 as opposed to in DH5 α , which shows higher fluorescence on a shorter timescale under anaerobic conditions. Error bars represent one standard deviation.

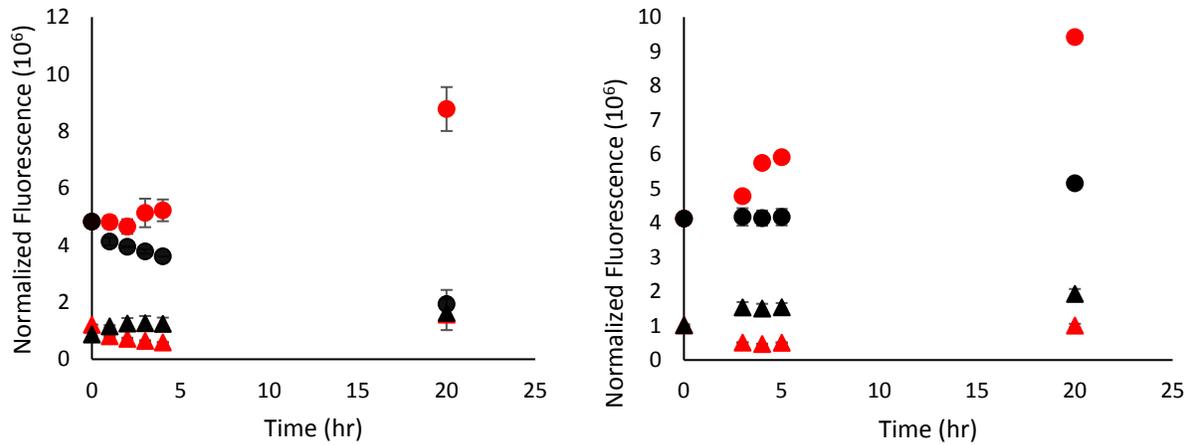


Figure 2. Red indicates aerobic conditions and black indicates anaerobic conditions. The circles represent the FNR plasmid and the triangles are the negative control. The two graphs show two different experiments in Wilms-Reuss Medium with the FNR promoter in MG1655. Results indicate that this promoter consistently has higher fluorescence in the presence of oxygen, while also having quite a bit of leaky fluorescence. Error bars represent one standard deviation.

pfl Promoter

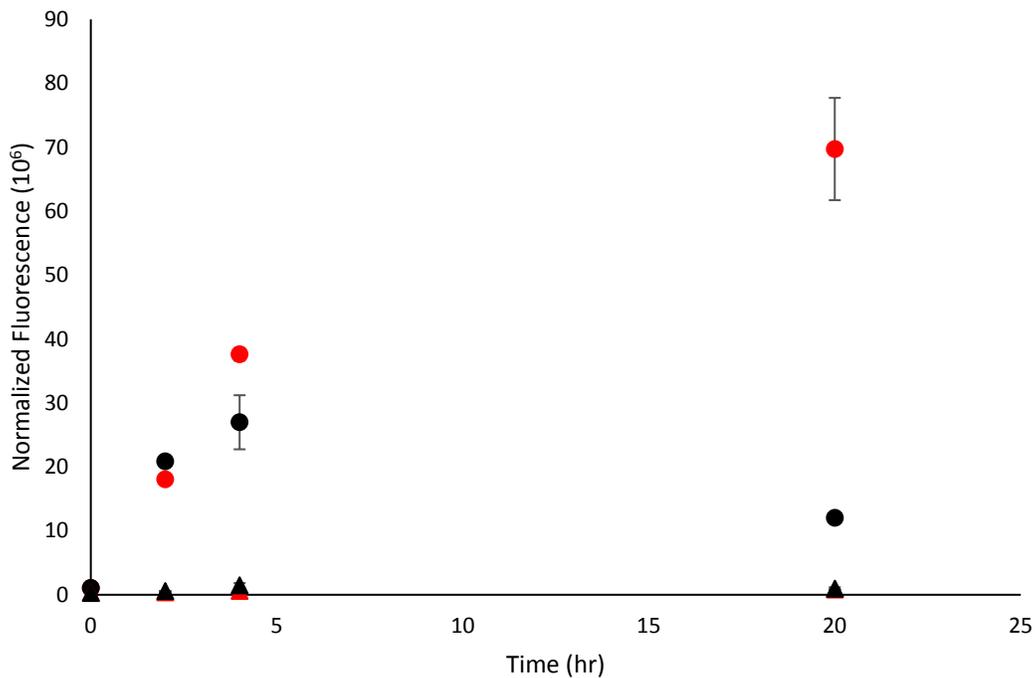


Figure 3. Red indicates aerobic conditions and black indicates anaerobic conditions. The circles represent the *pfl* plasmid and the triangles are the negative control. The experiment of the *pfl* promoter in DH5 α in LB medium resulted in data showing a higher fluorescence under aerobic conditions. Because of these results, this is the only experiment run on this specific promoter. Error bars represent one standard deviation.

Shown in Figure 3, there is little difference in the fluorescence of the bacteria under anaerobic conditions compared to aerobic conditions. This is likely due to the inability to achieve fastidious anaerobic conditions, only micro-aerobic conditions. Previous literature has found that the activity of the *pfl* promoter is significantly decreased, possibly over ten-fold, when not under strictly anaerobic conditions⁴. As a result, work on this *pfl* promoter was postponed in favor of the FNR activated promoter and the *gadB* promoter.

gadB Promoter

The *gadB* promoter showed the most promising results of the 3 promoters investigated. Consistent results were obtained when using the MG1655 strain in Wilms-Reuss medium (Figure 4). In both experiments, there was a higher level of GFP expression in the bacteria under anaerobic conditions. This indicates that distinctly higher levels of fluorescence can be reached in bacteria subjected to anaerobic conditions. However, consistent results were not achieved in LB medium (Figure 5). This suggests that there may be interactions in the media that are affecting the expression of GFP in these oxygen sensitive systems.

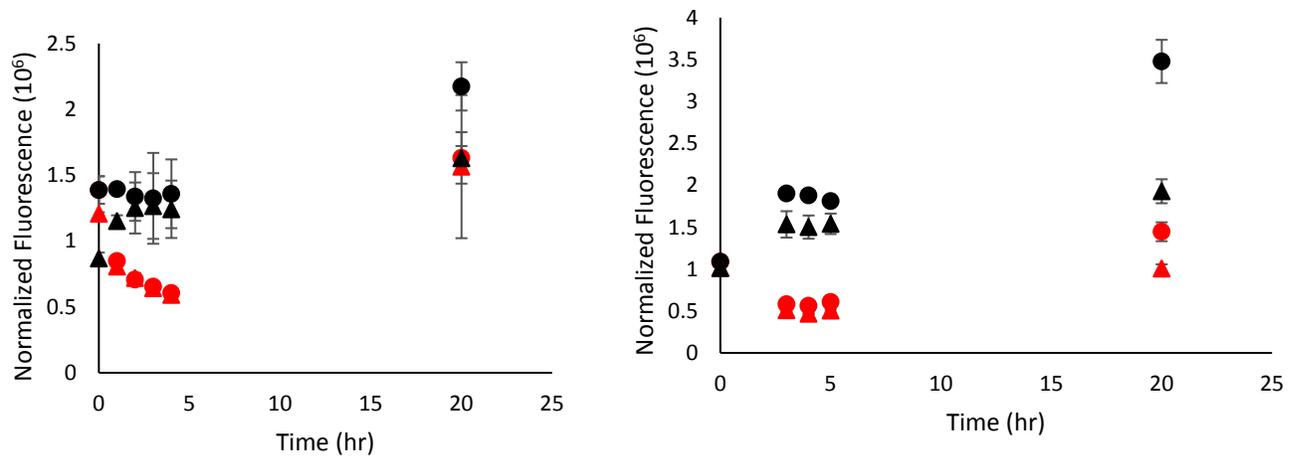


Figure 4. Red indicates aerobic conditions and black indicates anaerobic conditions. The circles represent the *gadB* plasmid and the triangles are the negative control. Two experiments were conducted with the *gadB* promoter in MG1655 using Wilms-Reuss medium. The bacteria showed consistently higher fluorescence when under anaerobic conditions compared to under aerobic conditions. Error bars represent one standard deviation.

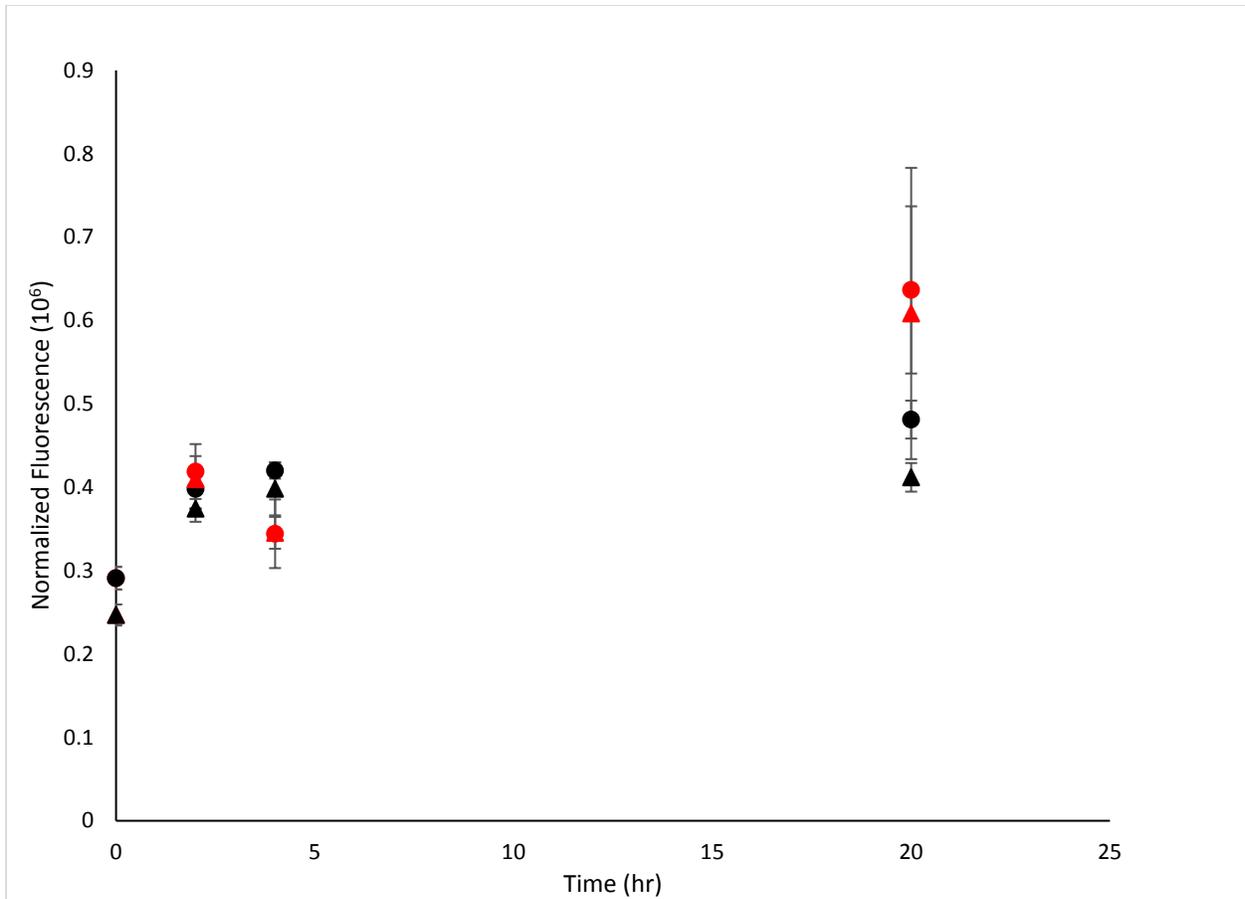


Figure 5. Red indicates aerobic conditions and black indicates anaerobic conditions. The circles represent the *gadB* plasmid and the triangles are the negative control. The experiment of the *gadB* promoter in MG1655 in LB medium resulted in data inconsistent with the two experiments done in Wilms-Reuss medium, indicating there was interaction between the promoter and the medium affecting GFP expression. Error bars represent one standard deviation.

Conclusions and future work

From the experiments run on the 3 promoters, *gadB* showed the most consistent and promising results. There was approximately a twofold increase in fluorescence between the anaerobic and aerobic conditions. With regards to the *pfl* promoter and the FNR protein activated promoter, their performance in both types of media under anaerobic conditions does not strongly indicate a higher level of selective protein expression. While the *gadB* promoter does show a higher level of GFP production under anaerobic conditions, it should be noted that the level of fluorescence is nearly comparable to the fluorescence level of the MG1655 strain without a plasmid. Therefore, this may not be a large enough of a difference to be noticeable in a bioreactor.

For future experiments, a different method of data collection may be useful in collecting data in shorter time intervals. For example, using flow cytometry may be helpful in collecting more data while the cells are still in exponential phase. Another option could be to use enzymes and running protein assays to test for promoter activity instead of fluorescence. In addition, testing other promoters can lead to better responses under anaerobic conditions. For example, the ArcA transcriptional regulator also activates the *cydAB* operon, which is more active under micro-aerobic conditions⁴. Another promoter to investigate could be the *yfiD* promoter, which is also activated under micro-aerobic conditions⁵. Such promoters may be more useful, possibly having a stronger response to being subjected to anaerobic conditions. This would allow for better detection of anaerobic spots in a bioreactor and possibly lead to development of improved mixing strategies.

Attachments

Fluorescence Study 070116 LB Medium.xlsx – Testing endpoints for fluorescence in FNR and *pfl* with DH5 α

Fluorescence Study 070716 LB Medium.xlsx – FNR and *pfl* in DH5 α

Fluorescence Study 071216 LB Medium.xlsx – Night experiment, FNR and *gadB* in DH5 α

Fluorescence Study 072416 Wilms Medium.xlsx – FNR and *gadB* in MG1655

Fluorescence Study 072616 Wilms Medium.xlsx – FNR and *gadB* repeat experiment in MG1655

Fluorescence Study 080116 LB Medium.xlsx – FNR and *gadB* in MG1655

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