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J. N. Andrews Honors Program
Andrews University

HONS 497
Honors Thesis

bcnABC operon transcription of *Pseudomonas putida* under varying iron and oxygen
concentrations and culture age

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March 31, 2014

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Primary Advisor Signature: _____

Department: Biology

Abstract

The species *Pseudomonas putida* produces hydrogen cyanide (HCN) through the transcription of the *bcnABC* operon. Previous research by Myrna Biswas (2010) demonstrated that microaerobic +FeCl₃ cultures had the greatest HCN production, but the effects of varying iron and oxygen levels on HCN production were unclear. Isaac Kim (2011) assessed HCN production of *P. putida* in sand versus soil using different iron concentrations and found that iron was necessary for HCN production. The purpose of this experiment was to determine how the *bcnABC* operon is affected by varying levels of iron and oxygen, and age of bacteria culture. To test this, *P. putida* was grown under four conditions: the presence of iron, the absence of iron, with aeration, and without aeration. At 8, 18, and 30 hours, the cultures were assessed for HCN production and cell density. HCN production was measured via bioluminescence; light emission occurs whenever HCN is produced because the modified form of *P. putida* contains the luciferase gene. The cell density was determined using spread plating. Bacterial cultures with iron and minimal aeration had higher bioluminescence levels, suggesting these conditions promote HCN production, but these results were not significant ($F_{(3,2)} = 0.561, p = 0.05$).

Introduction

The plant-associated bacterial genus *Pseudomonas* produces the secondary metabolite hydrogen cyanide (HCN). HCN is an inhibitor of the electron transport chain at cytochrome *c* oxidase and is poisonous to plants and mammals, resulting in instant death (Pessi and Haas 2000). While cyanide is toxic and is produced in the soil by *Pseudomonas*, plants and mammals are not usually affected by the poison (Blumer and Haas 2000a). In fact, the production of HCN by *Pseudomonas* helps to prevent plant-root fungal diseases such as tobacco black root (Blumer and Haas 2000a).

Furthermore, HCN suppresses seedling growth of a competitive weed species Velvetleaf (Myrna Biswas 2010).

Upon closer examination, the tightly regulated production of HCN synthase encoded by the *bcnABC* operon prevents the production of lethal doses of hydrogen cyanide. Hydrogen cyanide is produced by the enzyme HCN synthase (Blumer and Haas 2000b). The *bcnABC* genes form the *bcnABC* operon, which encodes for HCN synthase (Blumer and Haas 2000a). Figure 1 demonstrates the mechanism for HCN production.

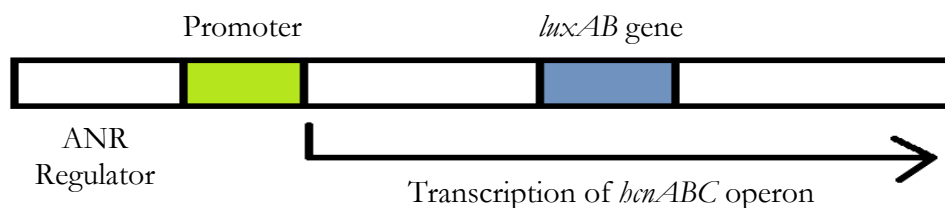


Figure 1. The *bcnABC* operon is a cluster of genes that encodes for HCN synthase, the enzyme responsible for HCN production. The modified form of *P. putida* contains the *luxAB* gene so that whenever HCN synthase is made, bioluminescence occurs. Upstream from the operon are the promoter and ANR regulator, which regulate the transcription of the *bcnABC* operon.

If HCN is produced, light will be emitted because of the modified *bcnABC* operon. This modified operon contains the luciferase gene, which endows light production when expressed. Whenever the *bcnABC* operon undergoes transcription, the luciferase gene will also be encoded, resulting in light emission.

The regulation of HCN can be categorized at two levels: first, at the level of transcription and secondly, at the enzymatic level (Blumer and Haas 2000a). Various substrates can influence HCN synthase activity and hence, affect hydrogen cyanide production. At the level of transcription, the *bcnABC* operon is influenced by two factors: the anaerobic regulator ANR and the global activator GacA (Blumer and Haas 2000b). ANR senses the intracellular levels of oxygen and can activate or repress the transcription of target genes, including the *bcnABC* genes encoding HCN

synthase (Blumer and Haas 2000b). Optimal production of HCN occurs with lower levels of oxygen (Blumer and Haas 2000b; Blumer and Haas 2000a). At low oxygen concentrations, less than 10 μM , ANR is converted to its activated form and turns on transcription of the *bcnABC* genes (Pessi and Haas 2000). When oxygen is present in high concentrations, ANR is inactivated and transcription of the *bcnABC* operon is prevented (Blumer and Haas 2000a).

In addition to low levels of oxygen, high levels of iron result in maximal HCN production (Blumer and Haas 2000a). Iron levels affect the regulation of the ANR protein; hence, under depleted iron supplies ANR is inactivated even though low concentrations of oxygen favor the active ANR form (Blumer and Haas 2000a). The global activator GacA is the second factor contributing to the regulation of the *bcnABC* operon. GacA is a component of the GacA/GacS system, acting as the response regulator for this system (Blumer and Haas 2000a). The system relies on a cell-density dependent mechanism termed 'quorum sensing', with GacA responding positively to higher cell densities (Pessi and Haas 2001). Greater cell densities activate GacA, which upregulates transcription of the *bcnABC* genes to produce HCN synthase and results in HCN production (Blumer and Haas 2000a).

My experiment investigated the effects of varying levels of iron and oxygen on the regulation of a modified version of the *bcnABC* operon in *P. putida* ATH2-1RI/9, more specifically through ANR and GacA. Another factor that was measured was the effect of the age of bacteria culture on HCN production. Blumer and Haas (2000a) and Pessi and Haas (2001) have researched HCN production by other bacterium of the genus *Pseudomonas*, such as *P. fluorescens* CHA0 and *P. aeruginosa*. Previous research by Myrna Biswas (2010) demonstrated that microaerobic +FeCl₃ stationary and log phase cultures had the greatest HCN production in *P. putida*, but the effects of varying iron and oxygen levels on HCN production were unclear. Isaac Kim (2011) assessed HCN production of *P. putida* in sand versus soil using different iron concentrations and found that iron

was necessary for HCN production, although his results were not significant. My research attempted to provide clarity on these previous studies for the species *P. putida* by testing the effects of iron and oxygen on HCN production in vitro.

Methodology

Culture Conditions and Enumeration

The bacterial strain *P. putida* ATH2-1RI/9 (containing the lux-modified *hcnABC* operon) was cultured in a shaking water bath at 28°C in King's B broth for 24 hours. Cells were recovered via centrifugation and resuspended in 0.5 mL of water. This cell suspension served as the inoculum, which was used in the test tubes during the experiment. My experiment investigated four conditions that could affect *P. putida*: the presence of iron, (+Fe), the absence of iron (-Fe), aeration (+O₂), and no aeration (-O₂). Each condition had three test tubes to ensure more than one sample per category. Every test tube contained 10 µL of inoculum in addition to 10 mL of MMC media. All test tubes contained an iron solution of 0.0003244 g FeCl₃/100 mL MMC media except for the -Fe test tubes. After 90 mL of the MMC media were used for the nine -Fe test tubes, 3 mL of iron solution was added to 300 mL of MMC media to use for the remaining 27 tubes.

For each condition, a total of nine test tubes were used, with three test tubes allotted for each of the time periods: 8, 18, and 30 hours. In all, there were 36 test tubes (see below).

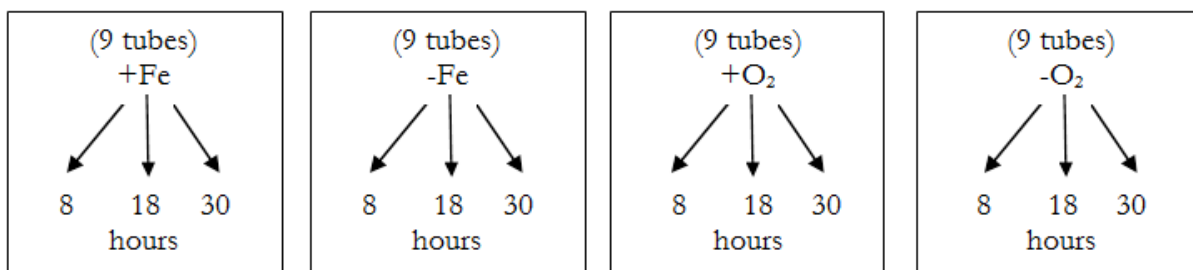


Figure 2. The bacterial culture was tested under four conditions, each represented by an individual square. For each condition, three tubes were measured at 8, 18, and 30 hours for a

total of nine test tubes. Light production and bacterial population determination were measured for all 36 test tubes at their respective times.

Each group of variables contained the iron solution except for the -Fe group, and each group was aerated except for the -O₂ group. The +Fe, -Fe, and +O₂ cultures were agitated in a shaking water bath at 28°C. The -O₂ group was incubated without shaking at 28°C. For each time period (8, 18, and 30 hours), bacterial populations were determined by serial dilution and spread plating in triplicate. Dilutions were performed by transferring 1 mL of culture into a test tube with 9 mL of 0.1% peptone, then transferring 1 mL into the next 9 mL test tube, and so on. For cultures at 8, 18, and 30 hours, there were 5, 6, and 7 dilutions performed respectively. The last three dilutions at each time period were used for spread plating in triplicate. 0.1 mL of culture from the dilution tubes were added to each plate.

Luminometry and Spectrophotometry

Each of the cultures were processed using a luminometer. 0.5 mL from the culture was added to a luminometer tube. A solution of 10 µL of 1% decyl alcohol in 100% alcohol was added to the luminometer tube. Luminescence readings were recorded for 2 minutes via the Spreadsheet Interface Software. The relative light units (RLU) produced over 2 minutes were then summed to represent light production in the culture. The turbidity or growth of each culture was determined by measuring the absorbance of the culture at 600 nm.

Statistics

Each treatment contained 3 samples and was spread in triplicate. These samples were averaged to derive the absorption values and CFU/mL ($\times 10^5$). The RLU for each sample was summed after 2 minutes and divided by the averaged CFU/mL ($\times 10^5$) from the plates. The RLU/CFU ($\times 10^5$) values were averaged from three experiments. To assess whether the treatments (+Fe, -Fe, +O₂, and -O₂) and age of bacteria culture (8, 18, and 30 hours) affected the results, a two-way ANOVA was utilized. The t-test was used to compare the +Fe and -Fe cultures at a certain time

period (for example, at 8 hours). The +O₂ and -O₂ cultures were also compared at a specific time period using the t-test. Comparisons were made within treatments using the t-test (for example, a comparison between 8 and 18 hours for the +Fe group).

Results

For cultures with iron and minimal aeration (-O₂ treatment), bioluminescence levels measured in RLU/CFU ($\times 10^5$) were higher than cultures with aeration (Figure 3). These results were not statistically significant ($F_{(3,2)} = 0.561$, $p = 0.05$). Bioluminescence levels were greatest for -O₂ cultures at all time periods, with the highest levels of bioluminescence occurring at 8 hours (Figure 3). This trend for the -O₂ cultures was not significant ($t_{(3)} = 0.447$, $p = 0.05$; $t_{(3)} = 0.067$, $p = 0.05$; $t_{(3)} = 0.395$, $p = 0.05$). The bioluminescence of -O₂ cultures decreased exponentially from 8 to 18 hours by a factor of 8 and decreased by half from 18 to 30 hours, but this was not statistically significant ($t_{(3)} = 0.393$, $p = 0.05$; $t_{(3)} = 0.182$, $p = 0.05$). The normalized data also showed that -O₂ cultures had greater bioluminescence than cultures with aeration, except the bioluminescence increased from 8 to 18 hours and decreased from 18 to 30 hours (Figure 4). The results from Figure 4 were not significant ($F_{(3,2)} = 1.293$, $p = 0.05$).

In Figure 3, the +Fe group had higher levels of bioluminescence overall in comparison to cultures without iron, but this was not statistically significant ($F_{(3,2)} = 0.561$, $p = 0.05$). At 8 hours, cultures without iron had greater bioluminescence levels than cultures with iron, but this was not significant ($t_{(3)} = 0.436$, $p = 0.05$). At 18 and 30 hours, cultures with iron had greater bioluminescence levels than cultures without iron, but this pattern was not significant ($t_{(3)} = 0.221$, $p = 0.05$; $t_{(3)} = 0.038$, $p = 0.05$). The normalized data showed that bioluminescence levels for the +Fe group were greater at all time periods, and that bioluminescence increased over time (Figure 4). These results were not significant ($F_{(3,2)} = 1.293$, $p = 0.05$). In Figure 3, the +Fe group had similar

levels of bioluminescence at 18 and 30 hours, although this trend was not significant ($t_{(3)} = 0.952$, $p = 0.05$). The -Fe cultures decreased from 8 to 18 hours and from 18 to 30 hours, but these results were not significant ($t_{(3)} = 0.357$, $p = 0.05$; $t_{(3)} = 0.292$, $p = 0.05$).

As the bacterial cell density (in CFU/mL $\times 10^5$) increased, the absorption also increased (Figure 4). This positive correlation was not significant ($r_{(35)} = 0.20$, $p = 0.05$). The +Fe treatment did not demonstrate increasing levels of bioluminescence with mature culture age, but these results were not statistically significant ($F_{(3,2)} = 0.561$, $p = 0.05$). The normalized data in Figure 4 showed that bioluminescence levels increased as bacterial density increased, but these results were not significant ($F_{(3,2)} = 1.293$, $p = 0.05$). For the -O₂ treatment, bioluminescence was greatest at 8 hours and decreased from 18 to 30 hours, but these results were not statistically significant ($F_{(3,2)} = 0.561$, $p = 0.05$).

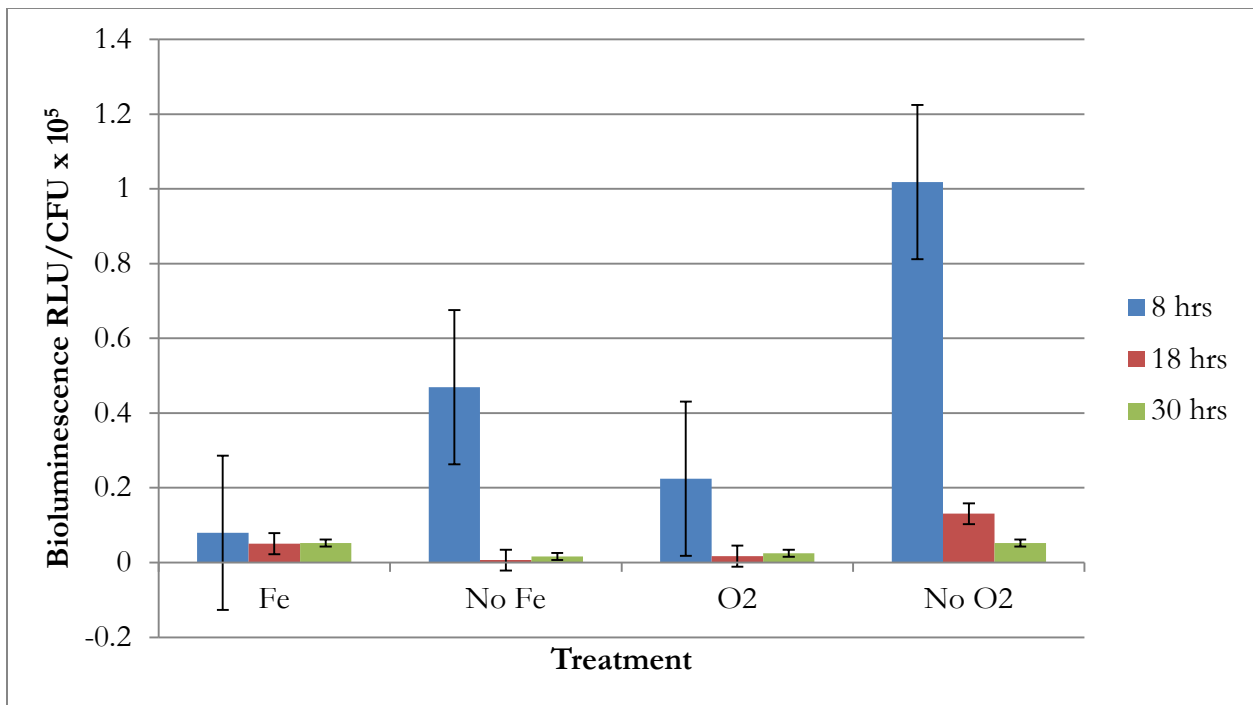


Figure 3. The graph above displays the average RLU/CFU $\times 10^5$ for three experiments. In each experiment, the relative light units (RLU) after two minutes were summed for a treatment at a particular time period. This was done for all three samples of the same treatment. The RLU of the three samples was divided by the same CFU/mL ($\times 10^5$) value and averaged together. These

calculations were done separately for each experiment. The graph above shows the average of three experiments with standard error bars. A two-way ANOVA statistical test revealed that there was no significant relationship between the different treatments and culture age on bioluminescence ($F_{(3,2)} = 0.561, p = 0.05$).

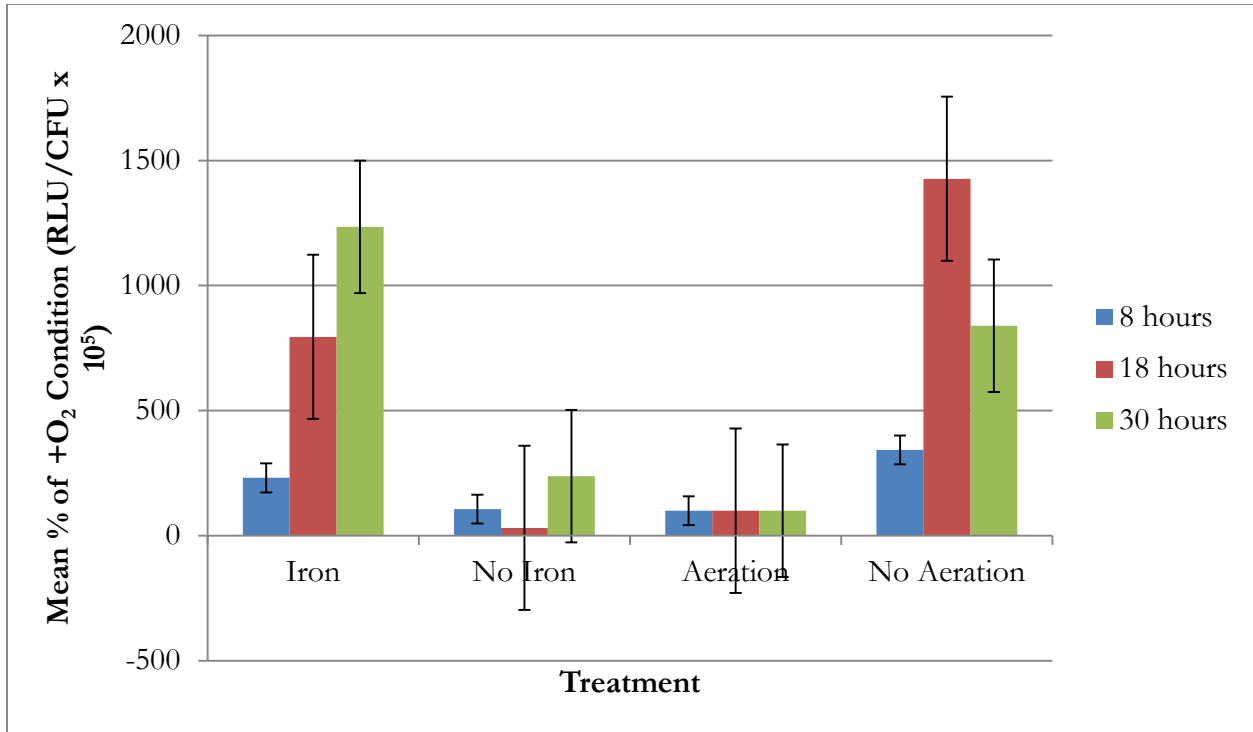


Figure 4. Each time period indicates the average relative light units (RLU) divided by the number of colony-forming units (CFU x 10⁵). Within each experiment, the RLU/CFU x 10⁵ values were averaged for each condition at their respective times. The RLU/CFU x 10⁵ values at 8, 18, and 30 hours for +O₂ cultures were set at 100%. At 8 hours, the RLU/CFU x 10⁵ values of all conditions were divided by the RLU/CFU x 10⁵ value of the +O₂ condition. At 18 hours, all RLU/CFU x 10⁵ values were divided by the value for the +O₂ condition. This calculation was repeated at 30 hours. After normalizing the data, values from the three experiments were averaged and graphed with standard error bars. A two-way ANOVA statistical test revealed that there was no significant relationship between the different treatments and culture age on bioluminescence ($F_{(3,2)} = 1.293, p = 0.05$).

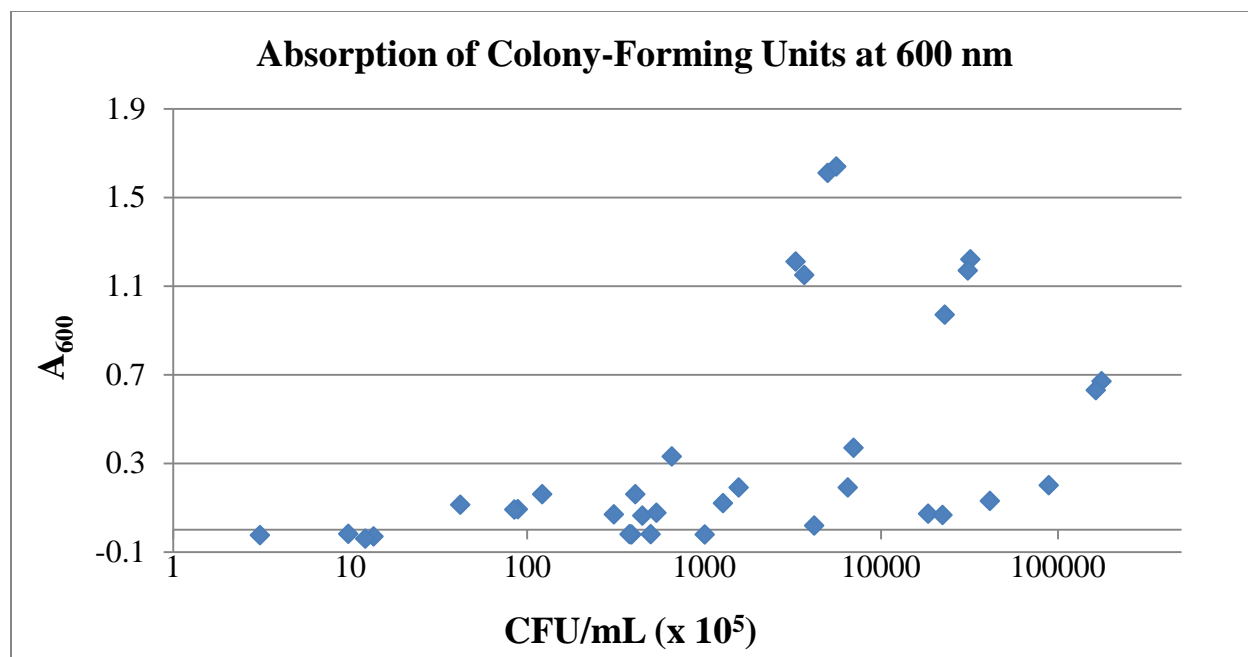


Figure 5. The absorption of each culture was measured at 600 nm for 8, 18, and 30 hours. The absorption values were plotted against the number of colony-forming units, and the x-axis was scaled by a logarithm of 10. The 3 samples from each condition were averaged and then plotted. The graph contains the absorbance from 3 experiments. As the CFU/mL ($\times 10^5$) increased, the absorption at 600 nm also increased, but this correlation was not significant ($r_{(35)} = 0.20$, $p = 0.05$).

Discussion

Varying levels of iron and oxygen were tested with control groups to demonstrate how their presence affected HCN production in culture. I predicted that bioluminescence levels would be greater for cultures with iron compared to those without iron. Previous research by Laville et al (1998) demonstrated that in the species *P. fluorescens* CHA0, iron was necessary for the activation of ANR. The activated ANR protein regulates the transcription of the *bcnABC* operon by turning on *bcnA⁺-lacZ* expression in the presence of iron. My results confirmed previous findings by Laville et al (1998) and showed that overall, iron was necessary for HCN production, although these results were not significant ($F_{(3,2)} = 0.561$, $p = 0.05$). Figure 3 indicated that cultures with iron had greater bioluminescence (and hence HCN production) compared to cultures without iron at 18 and 30 hours, but these results were not statistically significant ($t_{(3)} = 0.221$, $p = 0.05$; $t_{(3)} = 0.038$, $p = 0.05$).

The normalized data in Figure 4 showed that cultures with iron had greater levels of bioluminescence than iron-depleted cultures, although this trend was not significant ($F_{(3,2)} = 1.293$, $p = 0.05$). The variation between experiments was also noted by Myrna Biswas (2010), who found inconsistencies in her results: in culture, one of three experiments demonstrated a significant increase in bioluminescence in the presence of iron, but the other two experiments did not show significance. Difficulties with obtaining consistent results both in culture and the rhizosphere were reflected in previous research by Myrna Biswas (2010), Isaac Kim (2011), and my own research.

For conditions testing aeration, I predicted that optimal levels of HCN production would occur with high levels of iron and minimal aeration. Research by Blumer and Haas (2000a) demonstrated that the greatest levels of HCN production in *P. fluorescens* CHA0 occurred with high levels of iron and low levels of oxygen. In the absence of oxygen, the FNR/ANR recognition sequence in the -40 promoter region underwent transcription and the *bcnABC* operon was expressed (Blumer and Haas 2000a). Iron also influenced the expression of the FNR/ANR recognition sequence through the activation of ANR (Blumer and Haas 2000a). Pessi and Haas (2000) found that in *P. aeruginosa*, low oxygen levels activated ANR and enabled expression of the *bcnA* promoter, resulting in the activation of the T2 start site for transcription of the *bcnABC* operon. On the contrary, high levels of oxygen deactivated ANR and prevented transcription of the *bcnABC* genes (Blumer and Haas 2000a). Results from Figure 3 showed that high levels of iron and minimal aeration produced greater levels of HCN than cultures with high levels of iron and maximum aeration, but these results were not statistically significant ($F_{(3,2)} = 0.561$, $p = 0.05$).

For mature cultures, I expected the absorption and HCN production to increase because of increasing bacterial density. Figure 5 showed an increase in absorption as the bacterial density increased. Although there appeared to be a positive correlation, these results were not statistically significant ($r_{(35)} = 0.20$, $p = 0.05$). Previous research by Pessi and Haas (2001) confirmed that

hydrogen cyanide levels increased in *P. aeruginosa* PAO1 with exponential bacterial growth. Greater cell densities positively affected the transcription of the *bcnABC* genes via GacA, which in turn expressed the *rbII* gene (Pessi and Haas 2001). The *rbII* gene is responsible for the cell-density dependent mechanism of GacA, termed as ‘quorum sensing’ (Pessi and Haas 2001). Maximum bioluminescence occurred at 8 hours for cultures under the $-O_2$ condition and decreased at 18 and 30 hours, although these results were not significant ($F_{(3,2)} = 0.561, p = 0.05$). In Figure 4, the maximum bioluminescence levels occurred at 18 hours and decreased from 18 to 30 hours, but these results were not significant ($F_{(3,2)} = 1.293, p = 0.05$). One possible explanation for this trend is that initially, cell growth activated GacA which enabled transcription and expressed the *rbII* gene, but after reaching optimum levels of cell growth and *rbII* expression, transcription was turned off through quorum sensing via the global activator (Pessi and Haas 2000).

My research confirmed previous findings by Myrna Biswas (2010), Isaac Kim (2011), as well as other researchers who looked at the effects of iron and oxygen in different species from the genus *Pseudomonas*. The species *P. putida* requires iron for the transcription of the *bcnABC* operon, and my results showed that overall, cultures with iron had greater HCN production compared to iron-depleted, although these results were not statistically significant ($F_{(3,2)} = 0.561, p = 0.05$). In *P. putida*, the greatest levels of HCN production are expected to occur with high levels of iron and minimal aeration. My results supported this, but it was not significant ($F_{(3,2)} = 0.561, p = 0.05$). The relationship between bacterial density and HCN production was unclear for +Fe cultures in Figure 3 but showed that bioluminescence increased with culture age in Figure 4; these results were not significant ($F_{(3,2)} = 0.561, p = 0.05$; $F_{(3,2)} = 1.293, p = 0.05$). For $-O_2$ cultures, exponential cell growth initially lead to increased bioluminescence but then decreased; these results were not significant ($F_{(3,2)} = 0.561, p = 0.05$).

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