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Cyanide Production by Pseudomonas putida ATH2-1RI/9 Under Biofilm Conditions

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Honors Thesis

Cyanide Production by *Pseudomonas putida* ATH2-1RI/9 Under Biofilm Conditions

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Abstract

The genus *Pseudomonas* is a group of gram-negative bacteria having great ecological diversity. Some species produce HCN, which may harm plants. The purpose of this research is to determine whether a plant-associated strain of *P. putida* has the ability to produce cyanide under biofilm conditions. Bacterial production of HCN could prove useful in weed control strategies. To obtain these results the experiment involved a six-day experimental scheme. My findings indicate that *P. putida* does produce cyanide under biofilm conditions providing valuable information concerning cyanogenic bacteria especially in the *Pseudomonas* group.

Key Words

Pseudomonas, Pseudomonas putida, Cyanide, Biofilm conditions, Gram-negative bacteria, Weed control strategies.

Introduction

Pseudomonas putida is a gram-negative rod-shaped saprotrophic soil bacterium and has been proven useful in areas of bioremediation and biocotrol. It has been used as a soil inoculant to remedy naphthalene contaminated soils and is capable of converting styrene oil into biodegradable plastic and has shown potential as an effective antagonist of diseases such as fusarium and pythium. This particular strain of *P. putida* ATH2-1RI/9 has been studied and shown to produce cyanide. Cyanide production under biofilm conditions has been previously studied (Ryall et al., 2008) concerning the bacteria *Burkolderia cepacia* (BCC), which is said to be an opportunistic pathogen for people suffering from Cystic Fibrosis (CF). Cyanide production is of particular importance in that it is a potent inhibitor of cellular respiration and if this function is not available to the cells then energy will not be available for use throughout the body. *Psuedomonas aeruginosa* is another bacteria species involved in inflicting patients with CF lung and is already known to produce cyanide. Bcc can co-colonise with *P.aeruginosa* and in turn may be resistant to the toxic effects of cyanide as it itself is able to produce cyanide.

Production of cyanide is not an uncommon feature of bacteria in the *Pseudomonas* group but its production of cyanide under biofilm conditions is of particular interest to this research as a potential weed control strategy. Weed control strategies have previously been studied (Gurley and Zdor, 2004) where *Pseudomonas putida* and *Acidovorax delafieldii's* effects on rhizosphere colonization were studied and cyanide production and growth of velvetleaf and corn was examined. *P.putida* was shown to reduce velvetleaf growth and cyanide was recovered from the *P. putida* inoculated plants. The highest levels of rhizosphere HCN were found with *P. putida* ATH2-1RI/9-inoculated velvetleaf, ranging from approximately 53 to 68mM HCN/g root. The results of this experiment suggest that bacteria can discourage weed growth due to cyanide production. Also, previous work has been done using this particular strain of bacteria (Owen and

Zdor, 2000) where rhizobacteria were being studied for use as an alternative to herbicides as a biological control agent. The *Pseudomonas* group is of particular interest as it has shown promise in this area of control due to its ability to produce hydrogen cyanide (HCN). The gas negatively affects root metabolism and root growth and its availability to be produced has been correlated with precursors such as glycine, methionine and root exudates containing cyanogenic glycosides. *P. putida* ATH2-1RI/9, was also utilized in this experiment and was shown to produce higher levels of HCN with increased amounts of supplemental glycine.

Material and Methods

Six-day experimental scheme outlined:

Day 1: *Incubation*: Inoculate freshly grown *P. putida* cells in a mixture of Tryptic Soy Broth and glycine in an Erlenmeyer flask placed in a shaking water bath and incubated at 28^{o}C overnight.

Day 2: *Incubation*: Using 24 double-welled Erlenmeyer flasks, the outer well of each was filed with 10 ml of TSB and glycine and the inner well with 5ml of 1 M NaOH. Half of the total flasks present contained 100 6 mm glass beads, sufficient to cover the bottom of the flask. Each flask was inoculated with 10μl of *P.putida* and incubated at 28^{o}C.

Day 3-6: *Sample Harvest*: For four consecutive harvest days, three flasks with beads and three without were retrieved. Using the spectrometer placed 1ml of the NaOH from the inner well of each flask into a cuvette and obtained the absorbance of the sample. Used TSB to blank the spectrometer reading at 600 nm. Retrieved the rest of the NaOH and stored it in sterile tubes for cyanide analysis. Ran serial dilutions using 1ml of inoculum from flasks containing no beads in small dilution tubes filled with 9ml of 0.1% peptone and ran serial dilutions on flasks containing beads using 1 ml of inoculum after vortexing 10 beads in a 50 ml centrifuge tube for 2 min filled with 10 ml of 0.1% peptone. Spread plating was done in triplicate using 0.1ml from the last tube

of each dilution and incubated overnight. The number of colonies on each plate were then quantified and recorded.

Day 6: *Biofilm Production/ Cyanide quantification:* Determined whether biofilm production occurred using 20 beads from the flasks of the final harvest day and vortexed them in a 50 ml centrifuge tube filled with 20ml of crystal violet and letting it stand for 2 min. Then poured off the crystal violet and replaced it with 20ml of water and poured off the water immediately. Added 20 ml of decolorizer and vortexed for 2 min. Obtained absorbance readings for each sample using the spectrometer and placing the 1ml of the sample into a cuvette reading at 570 nm. Used decolorizer to blank the spectrometer. Conducted cyanide quantification using pyridine/succinimide reagents as outlined by Lambert et al. 1975. Anal. Chem 47:916.

Results

The results represent the varying amounts of *P.putida* populations in the presence or absence of glass beads as well as the cyanide levels recovered from the inoculated flasks with and without glass beads. Figure 1, 3, and 5 represent the average amount of colony forming units in the flask within one standard deviation. The general trend in all three graphs shows a greater amount of bacteria in flasks containing no beads as opposed to flasks containing beads. Figure 2, 4, and 6 represent the average amount of cyanide in the flasks within one standard deviation. While there is no specific trend in terms of whether cyanide was produced more over the course of the four days in flasks with or without beads, the

data shows that over time flasks containing cyanide produced a measurable amount more of cyanide.

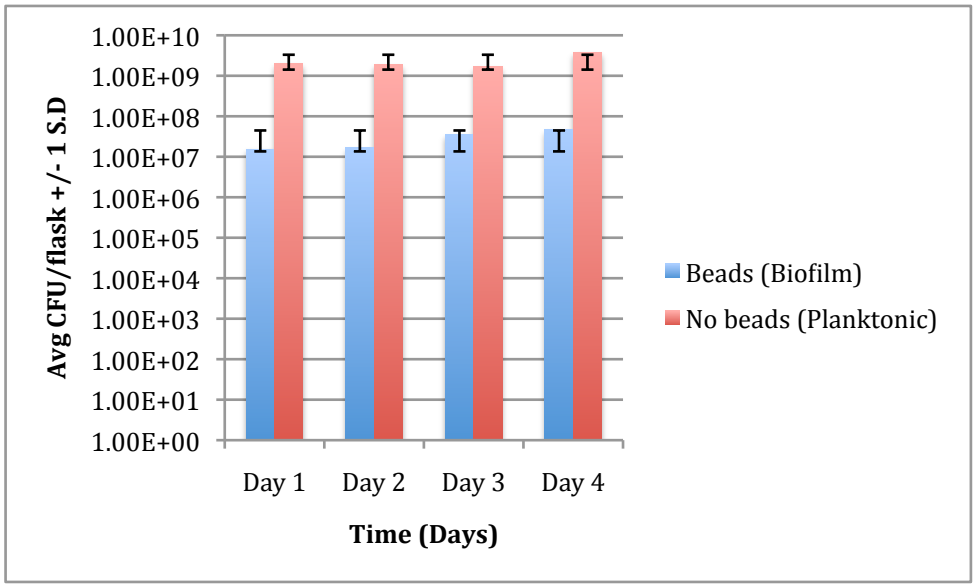

Figure 1: Experiment 1. *P. putida* populations in the presence or absence of glass beads over 4 days of growth. (Each mean is the average of 3 flasks with spread plates done in triplicate.)

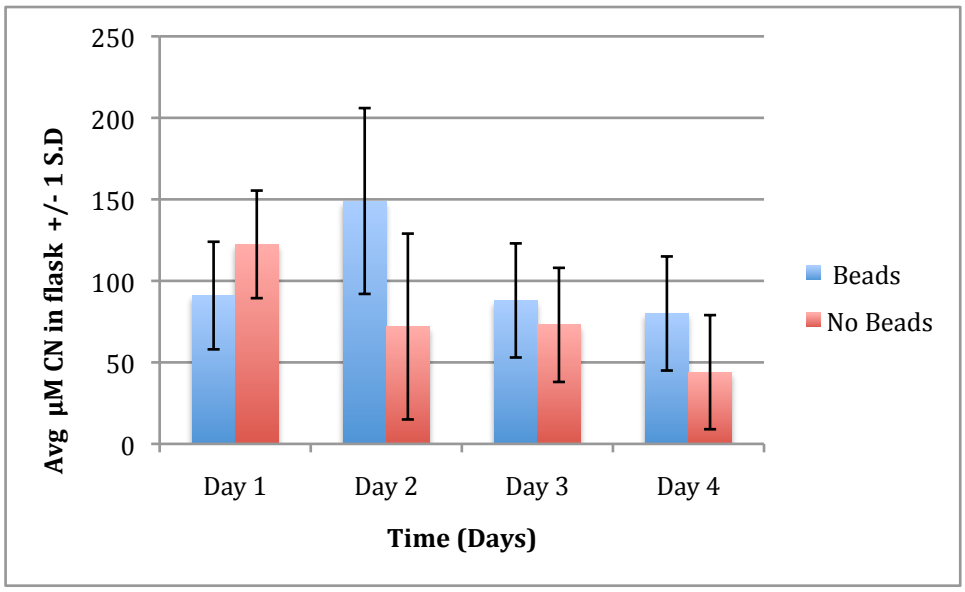

Figure 2: Experiment 1. Cyanide levels recovered from *P. putida* inoculated flasks with and without glass beads. (Each mean is the average of 3 flasks with cyanide determination done in triplicate.)

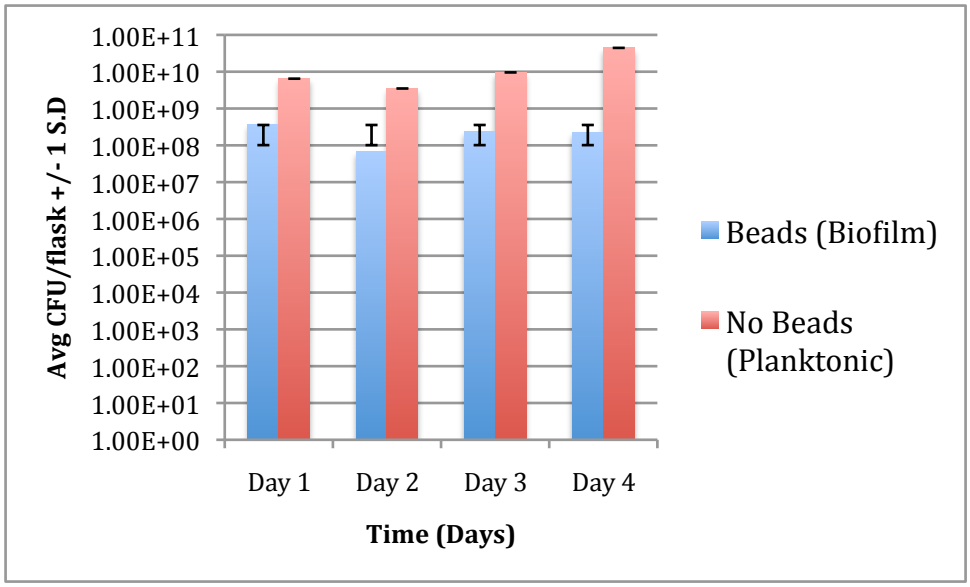

Figure 3: Experiment 2. *P. putida* populations in the presence or absence of glass beads over 4 days of growth. (Each mean is the average of 3 flasks with spread plates done in triplicate.

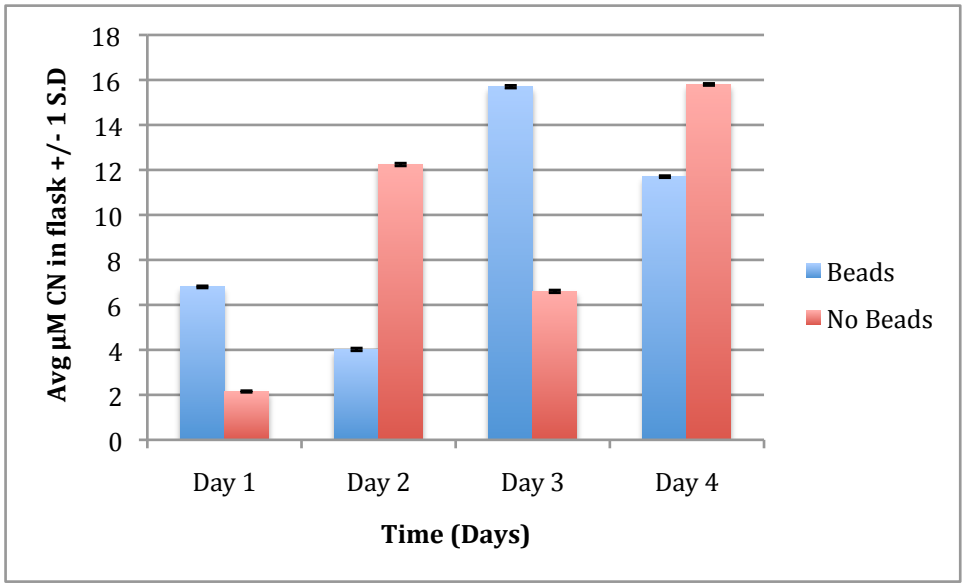

Figure 4: Experiment 2. Cyanide levels recovered from *P. putida* inoculated flasks with and without glass beads. (Each mean is the average of 3 flasks with cyanide determination done in triplicate.)

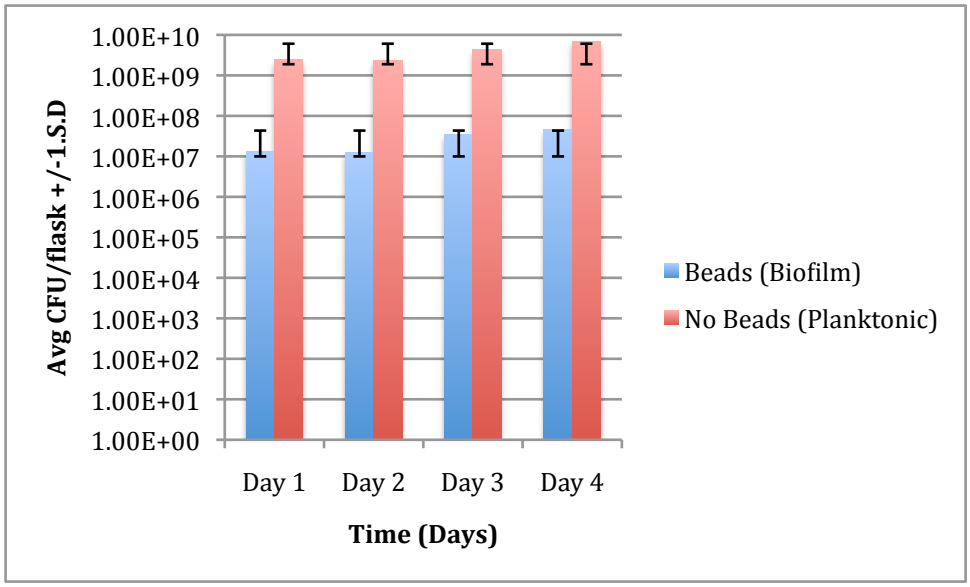

Figure 5. Experiment 3. *P. putida* populations in the presence or absence of glass beads over 4 days of growth. (Each mean is the average of 3 flasks with spread plates done in triplicate.

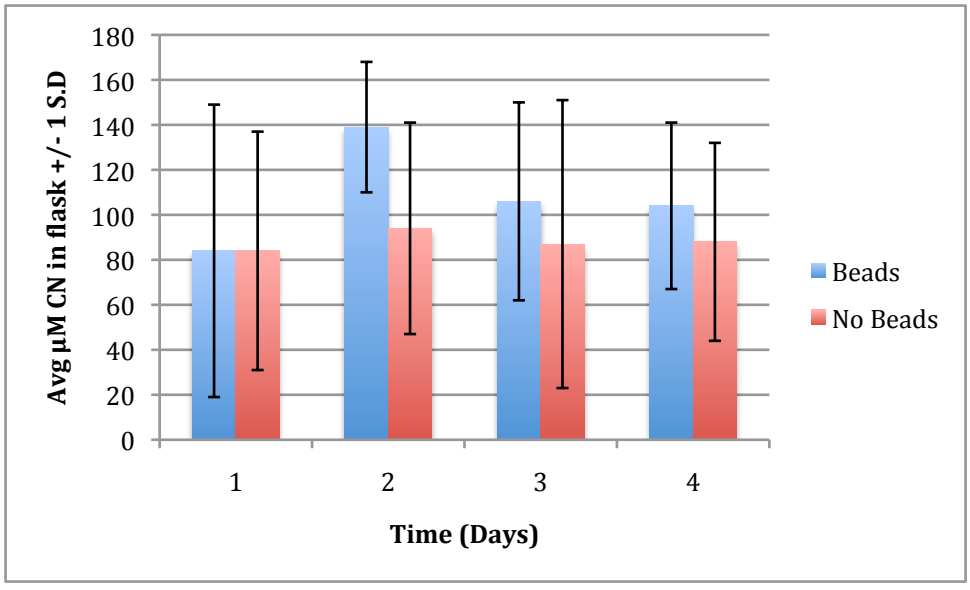

Figure 6. Experiment 3. Cyanide levels recovered from *P. putida* inoculated flasks with and without glass beads. (Each mean is the average of 3 flasks with cyanide determination done in triplicate.)

Discussion

Analysis of the data indicates that P. putida does produce cyanide under biofilm conditions. Prior work suggests P. putida forms biofilms (Jakovleva et al, 2011). The necessity of a surface for the bacteria to attach for cyanide production to has been demonstrated by Ryall et al (2008) and their study of *B. cenocepacia.* These data are consistent with what is expected of bacteria in the Pseudomonas group (Gilchrist et al, 2011). *P.putida* ATH2-1RI/9 does form biofilms as detected using crystal violet staining and CN is detected under these same conditions. If CN is important in weed suppression by *P. putida* then conditions that enhance biofilms may improve weed control by this bacterium (Owen and Zdor, 2000). The crystal violet data did not provide any significant information to be reported as to whether or not a biofilm was actually formed. This is probably due to the fact that in the outlined technique for rinsing the beads with decolorizer and water, there was still a residual amount of the crystal violet stain on the beads, which probably affected the way the spectrometer measured the level of absorbance of the liquid drawn off of the beads. The cyanide data does show, however, that there was a significant amount of cyanide produced by flasks containing beads suggesting that there must have been a certain amount of biofilm produced n order for the cyanide to be produced.

Acknowledgements

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