

Detection of Para-Cymene, a Microbial-Generated Volatile Organic Compound (MVOC), By Use of a Bioluminescent Biosensor, a Proof of Concept

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Abstract

All environments inhabited by humans contain microbes. The detection and elimination of harmful fungi and bacteria in closed system environments such as spacecraft is of vital importance. Biological waste products of microbes can cause "sick building syndrome," structural damage, and pose a fire hazard. Traditional means of detecting and quantifying these microbes, GC/MS and HPLC, are too bulky and labor intensive to operate in space. Biosensors provide an alternative to conventional instrumentation. This study examined the use of bioluminescent biosensors for the detection and quantification of microbial-generated volatile organic compounds (MVOC's). A strain of *Pseudomonas fluorescens* containing the *lux* operon from *Vibrio fischeri* was suspended in an alginate bead and exposed to para-cymene. The MVOC, para-cymene, can be metabolized by *P. fluorescens*. This strain has been engineered to stimulate activation of the *lux* operon resulting in the production of bioluminescence with para-cymene metabolism. A non-linear qualitative but perhaps non-quantifiable relationship was observed between para-cymene concentration and electric current generated by bioluminescence.

Introduction

This work was in support of Dr. Jay Garland's project "Calibration and Stability Testing of a Microbial Volatile Organic Contaminant (MVOC) Biosensor."

Human habitation is always concomitant with microbial habitation. These microbes, including fungi and bacteria, produce metabolic wastes. On Earth these wastes can be detrimental but the presence of an open system mitigates these effects. In closed systems such as the International Space Station, shuttle orbiters, and any future manned Mars missions, the potential for microbial induced problems is serious.

A principal group of metabolic waste products from fungi and some bacteria is volatile organic compounds, (VOC's).^{1,2} Many of these MVOC's, especially terpenes, are unique products of specific fungi or bacteria.¹ On Earth these compounds have been found to be responsible for "sick building syndrome" as well as acting as a corrosive agent upon structural materials.² In space these problems will be more severe. Most organic compounds are combustible, which produces a potential for fires in the high oxygen environments encountered in space vehicles. The traditional means of determining the presence and identity of these compounds has been by use of gas chromatography-mass spectrometry, (GC/MS), or high performance liquid chromatography, (HPLC).^{1,2,3} Both of these systems, while very accurate, are bulky and high mass, which are limiting factors with current propulsion technology. Additionally, these systems require extensive crew time and do not even provide real time detection or analysis of bio-contaminants. In spacecraft air filtration and surface disinfection will be key in eliminating MVOC's and microbial growth. Early detection of these compounds is then the critical issue. Chemical weapon sensing is another area in which simply determining presence or absence of a compound is important. Both the American and the British military have developed biosensors that can detect minute quantities of chemical agents.^{3,4} The British biosensor is based on bioluminescence of a genetically modified bacterium. A similar system could be developed for real time early detection of MVOC biocontamination.

Bioluminescence is the process of light emission by a living organism. In bacteria it is produced by the oxidation of reduced flavin mononucleotide, (FMNH₂), and a fatty aldehyde in the presence of molecular oxygen catalyzed by the enzyme luciferase.⁵ Bioluminescence is extremely rare in terrestrial bacteria but more common in marine species. The genes responsible for bioluminescence in one aquatic bacterium, *Vibrio fischeri*, have a particularly well-characterized sequence known as the *lux* gene cassette.^{4,5,6} This cassette, or operon, consists of five structural genes, *luxCDABE*, which encode for both luciferase and a multi-enzyme complex that catalyzes aldehyde biosynthesis.^{2,5,6} The cassette is fully self-contained, meaning no exogenous substrate nor cell lysis is required to achieve bioluminescence.^{2,6} A

recombinant, man-made, version of this gene cassette has been successfully inserted in a number of plasmid cloning vectors with subsequent transformation into and functional expression by several strains of *Escheria coli*, *Psuedomonas aeruginosa* and *P. fluorescens*.^{6,7}

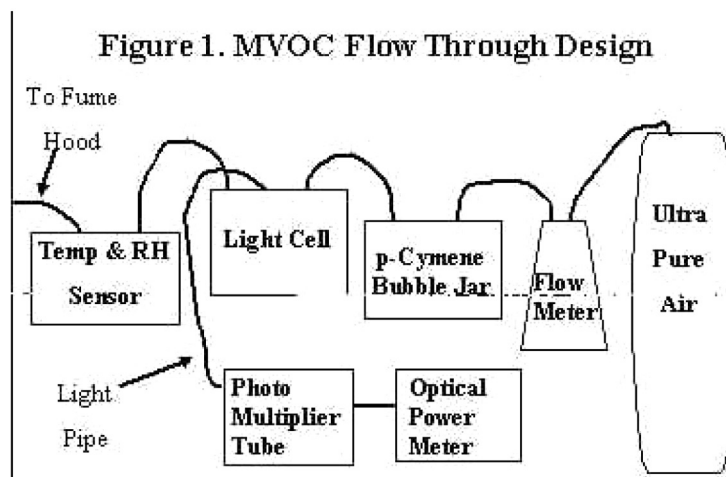
Previous Work

The Center for Environmental Biotechnology (CEB) at the University of Tennessee-Knoxville has engineered a bioluminescent strain of *P. fluorescens* and developed a method by which this bacteria may be incorporated into a biosensor.^{2,7,8} The *P. fluorescens* contains plasmid vector pUT mini-Tn5-*cym-lux* with a *cym* promoter, the *lux* gene cassette and genes coding for resistance to both kanamycin and ampicillin. The biosensor was created by suspending the bacteria in a droplet of sodium alginate that was then hardened in a strontium chloride solution.^{8,9} A metabolic pathway using benzene ring containing compounds is activated in the presence of para-cymene in this bacterium, which triggers bioluminescence.¹²

Dr. Val Krumins of the NASA Life Sciences Support Contract (LSSC) division of Dynamac Corporation had developed a concept for a flow through analysis system for the biosensor. Drs. D. Kong and L. Levine, also of Dynamac Corporation LSSC division have conducted GC/MS studies of para-cymene at varying concentrations for calibration of the biosensor.

Methods and Materials

A flow through analysis system was constructed (figure 1). A flow-regulated stream of ultra pure air was bubbled through a solution of para-cymene, ethanol, and distilled water that half filled a 500mL jar. The flow of bubbled air was mixed with and forced the volatilized para-cymene above the solution to flow out of the jar and into the biosensor cell. The biosensor chamber, a light tight jar, contained a fitting for holding the biosensor bead and for holding the tip of the fiber optic light pipe a fixed distance directly above the bead. The biosensor chamber also contained inlet and outlet ports as well as a septum for drawing off headspace gas for GC / MS calibration. Bioluminescence was measured using a fiber optic light pipe (Oriell 77568) coupled with a photo-multiplier tube (Oriell 77340) connected to a multifunctional optical power meter (Oriell 70310) for read out measurements. Downstream of the biosensor chamber was a final jar for measurement of relative humidity and temperature with an outlet to a fume hood. All fittings upstream and within the biosensor chamber were of non-reactive stainless steel or Teflon, while those downstream were a mixture of Teflon, PVC, and surgical tubing. Due to difficulties with rapid loss of para-cymene concentration most measurements were taken in a closed cell while a modified flow through system was engineered. A dark room was constructed using vinyl sheeting and duct tape within the LSSC physiology lab in Hangar L, Cape Canaveral Air Force Station.



The *P. fluorescens* for the biosensor was grown in yeast extract-polypeptone-glucose (YEPG) media treated with kanamycin (Kn), for plasmid selection, overnight at 30°C. The bacteria-YEPG-Kn culture was then be added at a ratio

of one to ten to fresh YEPG and cultured to an optical density of 0.8 measured on a spectrophotometer at 546nm. It was diluted with an equal volume of minimal salts media (MSM), which consisted of 0.4 mM $MgSO_4 \cdot 7H_2O$ and 2.5 mM NH_4NO_3 . The diluted culture was then added one to two to a sodium alginate solution, mixed on ice, and pushed through a wide bore syringe falling drop wise into a 0.1 M strontium chloride solution. The drops solidified into beads in the $SrCl_2$ solution with bead hardness increased by longer duration within the bath.⁸

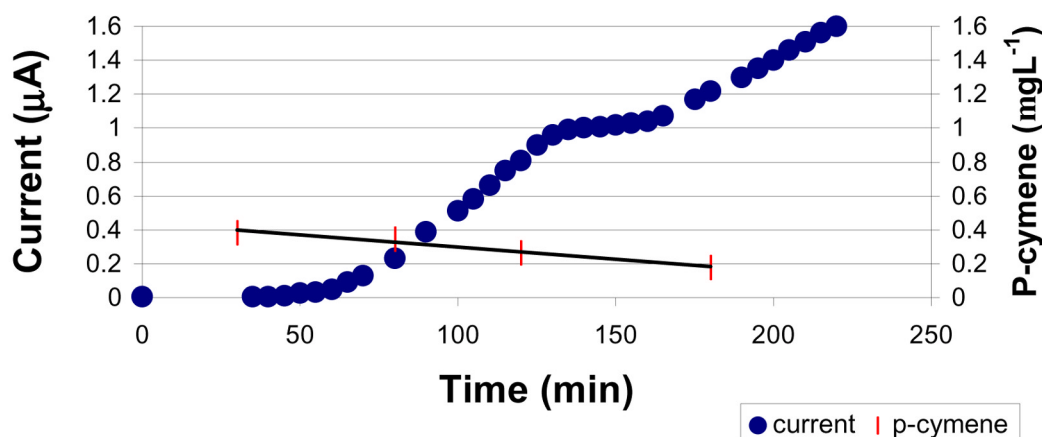
The hardened beads were placed in the biosensor cell and exposed to para-cymene vapor. The bioluminescence was measured and compared with GC / MS analysis of the headspace gas to derive a calibration model. Higher concentrations of para-cymene were drawn off by syringe. In preparation for lower threshold analysis of para-cymene, below the limit of syringe draws, calibrations of Solid Phase Micro Extraction (SPME) were preformed. SPME uses a small diameter fused silica fiber coated with a polymeric stationary phase to extract analyte by adsorption.¹⁰ The analyte can be easily desorbed thermally in the injection port of a GC/MS. SPME has been shown to be up to 100 times more sensitive than conventional syringe headspace analysis.¹¹

Lack of measurable bioluminescence initially spurred heightened asepsis, varied bead holding devices, and plate lawn colony measurements to no avail. Receipt of a second culture from the CEB allowed work to progress.

Results

No experiment conducted with *P. fluorescens* culture 1 yielded luminescence above the background noise threshold.

P. fluorescens culture 2 was analyzed by the plate method and GCMS data was obtained for the headspace para-cymene. After a 45 minute lag the biosensor displayed exponential like increase in current for 80 minutes. This was followed by a 35 minute plateau and ended with a 45 minute linear growth period (figure 2). Over the course of the experiment the average headspace para-cymene concentration decreased from 0.19 mgL^{-1} to 0.088 mgL^{-1} (figure 2).



Discussion

All work with culture 1 proved to be controls and checks against contamination. The immediate response obtained with culture 2 validated the procedures used and indicated that culture 1 was defective for the *luxCDABE* operon. This deficiency may have been caused by selection of a colony that developed spontaneous resistance to kanamycin without the recombinant insert. The results shown here are from the plate method but this work will be incorporated into further work with culture 2 using the bead method. The bead method should lead to a product that can be integrated onto a biosensor chip while the plate method merely demonstrated the viability of the inserted bioluminescent operon.

The continued increase in current generated by the biosensor even after nearly three and a half hours supports the hypothesis that para-cymene can be detected at concentrations much lower than the 0.088 mgL^{-1} used, hopefully with a threshold in the low ppb range. The 50 minute lag between exposure and the onset of measurable response indicates

that the biosensor is hindered in delivering an acute response but this should not prove unworkable at the concentrations being detected. Additionally, as crew time is a major constraint on spacecraft, the ability to qualitatively monitor for contaminants with minimal crew involvement will be a great boon.

Implications and Further Research

When it is felt that an adequate calibration model for the correlation of bioluminescence to concentration, or simply lower threshold detection if correlation is not possible, has been achieved the known para-cymene bubble chamber will be replaced with a fungal bubble chamber. The fungal bubble chamber will contain a culture of *Penicillium roqueforti* in sucrose-yeast-extract solution (SYES). SYES contains 20g yeast, 150g sucrose, and 20g agar per liter with an additional 5 ppm $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 10 ppm $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The culture of *P. roqueforti* will be grown until a measurable amount of para-cymene is detected by the biosensor.

This should lead to a detection system for para-cymene to be used in air quality measurements on spacecraft. Further research to develop additional biosensors specific for other uniquely linked metabolic wastes could lead to an integrated biosensor array for detection of biocontaminants.

Correspondence with individuals still involved with the project indicates that the non-linear relationship continues to be observed, that the detection threshold is approaching acceptable levels, and that a working flow through system has been implemented.

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