ETHYLENE SYNTHESIS BY GENETICALLY MODIFIED *RHODOCOCCUS OPACUS* PD630 ON SYNTHETIC HUMAN URINE[1]

A. Williams and K. Wawrousek, PhD.[2]

Abstract: To accommodate NASA's Center for the Utilization of Biological Engineering in Space objectives for genetically modified organisms to produce materials and food sources required for Mars colonization, we are modifying a bacterium to produce ethylene from genetic modification. Our non-model bacterium, *Rhodococcus opacus PD630*, is a bacterium that has been proven in the literature to be metabolically diverse enough to eat urea, a compound largely found in human urine. It has also been found to continue to grow at the same time as it is accumulating fatty acids, a trait that is useful for our research as it would allow fatty acids to be convertible in large quantities to ethylene if provided the correct gene through plasmid introduction. Growth curve studies on Luria-Bertani Broth and synthetic human urine were conducted on *R. opacus PD630* to establish the growth behavior of the wild-type bacteria and then a crafted plasmid consisting of a pBAV1K backbone, efe gene, and PsbA promoter-Ribosome binding site was introduced into competent cells. Post plasmid introduction, growth media studies and plasmid identification through selective digestion would be conducted to ensure pure bacterial colonies for eventual ethylene production. Ethylene production would then be promoted through bacteria growth in synthetic human urine and kanamycin, later to be detected via gas chromatography.

Additional Key Words: *Rhodococcus opacus*, genetic modification, ethylene production, human urine, Mars colonization, NASA, fatty acid accumulation, efe gene.

[1] To be presented via an academic talk at the 2021 AIChE Annual Meeting on November 7-11 in Boston, Massachusetts and at the University of Wyoming's Undergraduate Research Day in December 2021 via poster.

[2] Karen Wawrousek, Ph.D. is an Assistant Professor in the Department of Chemical Engineering, University of Wyoming, Laramie WY 82071. Alexandria Williams is a Senior Undergraduate Researcher, Dept. of Chemical Engineering, University of Wyoming, pursuing a Ph.D. in Chemical Engineering and Biomedical Engineering.

Introduction

There has been a considerable amount of interest established through the National Aeronautics and Space Administration's Center for the Utilization of Biological Engineering in Space for genetically modified organisms to produce materials and food sources required for Mars colonization. Transporting building material is one of the more relatively higher expenses in space travel, averaging a cost per pound of loaded material of 10,000 USD in 2008 (NASA 2008). To reduce the impact cost has on colonization, we propose to introduce *Rhodococcus opacus* PD630 to a gene that would convert fatty acid digestion precursor molecules like α-ketoglutarate into ethylene. Then, this ethylene be harvested for polyethylene production from common waste material that can be found during a result of the voyage as well as colonization. The resultant polyethylene produced from our ethylene could be utilized for building material in 3-D printing of crucial parts during space travel and foundational colonization of Mars. *Rhodococcus opacus* PD630 is one of the most studied *Rhodococcus opacus* strains and has been proven in the literature to be not only metabolically plastic, but oleaginous, producing triacylglycerol and wax esters from a wide range of material, including but not limited to glucose, acetate, hexadecane, and urea while continuing to grow relatively unhindered by fatty acid accumulation (Castro et al. 2016). A urea stock found in space travel that could be considered as a safe housing environment for the bacteria would be sterilized astronaut urine, a material produced naturally from the living that is often discarded as, without filtration, a waste material.

With these characteristics in mind, we had designed several experiments to demonstrate *Rhodococcus opacus* PD630's ability to grow on synthetic human urine to replicate the bacteria's response to sterile astronaut urine based on a procedure presented in an article called A New Artificial Urine Protocol to Better Imitate Human Urine by Sarigul et al. Furthermore, experiments on electroporation methods for the *Rhodococcus opacus* PD630 cells and cloning a plasmid that would be capable for converting fatty acid precursor molecules to ethylene were established to lay the foundation for future ethylene detection experiments on the genetically modified *Rhodococcus opacus* PD630 via gas chromatography.

Methods

Development of Both Growth Media

Luria-Bertani (LB) broth was used as a complete control media. To make a non-supplemented, liquid stock of this media, 20 g of LB Broth powder per liter of Deionized (DI) Water was autoclaved at 121 Celsius for 18 minutes and let cool to room temperature before storage on a room temperature shelf. Any significant change (>5%) in the OD of the liquid was considered contaminated and disposed of through the addition of bactericide-grade bleach before it could impact any experimental data. LB-Agar plates were made using 20 g LB Broth powder and 18 g Bacterial Agar per liter of DI Water and autoclaved for 18 minutes at 121 Celsius. The LB-Agar media was then allowed to cool until a gloved hand could hold the bottle for 3 seconds without sensory objection. At this point, LB-Kan (Luria-Bertani Broth and Kanamycin) selective media plates can be made by adding kanamycin until the mixture has a kanamycin concentration of 50

µg/mL or remain as non-selective LB-Agar media and be poured into labeled Petri dishes until halfway full.

Synthetic human urine, called urea media in all experimental data, was used as the experimental media. To produce synthetic urine that adequately represents the chemical properties of real human urinary excretion, careful replication of artificial urine produced by Sarigul et al. was created. This media is composed of 0.17 g Na2SO4, 0.025 g C5H4N4O3, 0.072 g Na3C6H5O7*2H2O, 0.0881 g C4H7N3O, 1.5 g CH4N2O, 0.2308 g KCl, 0.1756 g NaCl, 0.0185 g CaCl2, 0.1266 g NH4Cl, 0.0035 g K2C2O4*2H2O, 0.1082 g MgSO4*7H2O, 0.2912 g NaH2PO4*2H2O, and 0.0831 g Na2HPO4*2H2O per 100 mL of DI water with a magnetic stirrer rotating at 450 rpm. Due to the high salinity of the media, the mixture was sterile filtered into an autoclaved bottle and capped through sterile technique. The fluid bottle would then be placed in an incubator overnight at 37.5 Celsius before use or storage. Urea-Agar plates could then be made with the incubated media by introducing autoclaved agar media composed of 18 g of Bacterial Agar per liter of DI water in a 50-50 solution by volume of agar media to synthetic human urine. Kanamycin could then be introduced once the bottle can be held for 3 seconds with a gloved hand without pain to a concentration of 50 µg Kanamycin /mL of media or poured directly into Petri dishes until filled halfway.

Agar plates of both media were allowed to set until condensation ceased and stored lid-side down in aluminum foil at 4 Celsius until inoculated with bacteria. All inoculated plates marked for storage were wrapped in Parafilm lid-side down and stored at 4 Celsius where their colonies would be marked with marker and harvested for testing or allowed to grow further in an incubator at 30 Celsius for *Rhodococcus opacus* PD630 or at 37 Celsius for *Escherichia coli*. Any plate older than 2 weeks was discarded into the biohazardous waste to be disposed of properly and a sample of all plasmid variants produced before the 2-week expiration of the plate was grown in a 2 mL 8-hour growth of LB media and placed in a 10 vol% glycerol solution of bacteria inoculated media and chilled at -80 Celsius. Glycerol stock storage was ensured to allow replication of experiments and mass production of plasmid components unless the bacteria was previously electroporated where only growth and digestion identity experiments could be run.

Growth Study Procedure and Conditions for Termination

Multiple growth study experiments were conducted on *Rhodococcus opacus* PD630 with the same base procedure. Seed samples of *Rhodococcus opacus* PD630 for both the LB control vials and urea experimental vials were prepared using 6 mL of LB and 6 mL of synthetic human urine in capped conical tubes and inoculated with 3 loops of *Rhodococcus opacus* PD630 glycerol stock where they would be grown for at least 8 hours at 30 Celsius and 150 rpm. Once grown, the 2 mL of the seed of matching growth media was introduced into 90 mL of their respective growth media in three 500 mL Erlenmeyer flasks and gently swirled until evenly dispersed. These vials were then labeled with the date, initials, species, growth media, growth conditions, growth curve name, and #1, 2, or 3 to distinguish the vials and data points between all 3 sample vials for a specific media. The incubators were then set for their specified temperature and rotation speed, where their optical density (OD) was measured against a blank solution of its respective growth media in a 10 mm cuvette through a spectrophotometer at 600 nm and recorded in an excel document. The growth curves were grown at 30 Celsius and 105 rpm to

allow optimized growth of the bacterial specimen. Data points were taken every 6 hours to promote accurate evaluations of the growth rate for *Rhodococcus opacus* PD630 in both growth mediums.

The growth experiments were terminated once 3 OD values differing in less than 5% change occurred in sequence, suggesting the being of stationary phase in the growth cycle of the test bacteria in the growth media. Flasks to be discarded were introduced to bactericide-grade bleach and thoroughly washed with lab-grade glass cleaner. The Erlenmeyer flasks were allowed to dry completely and then autoclaved to ensure sterility. Furthermore, all data points collected were placed into scatter plots where their values and growth patterns could be compared between Luria-Bertani Broth and synthetic human urine can be compared directly.

Isolation of Plasmid Components

Using sterile technique, LB-Kan plates were inoculated with *Escherichia coli* containing an efe gene, 1071 bp in length, and grown in a non-rotating incubator at 37 Celsius overnight. Colonies were then harvested and introduced in 2 mL of 50 µg Kanamycin/ mL LB tubes and incubated at 37 Celsius and 150 rpm overnight. The resultant cells were harvested and the plasmid containing the efe gene was extracted using a GenElute™ Plasmid Miniprep Kit with DI Water as the final elute solution. Post extraction, the plasmid was stored at -20 Celsius until used in a 50 µL digestion composed of 5 µL of 10x rCutsmart buffer, 1 µL of XbaI restriction enzyme, 1 µL of SpeI restriction enzyme, and 43 µL of plasmid DNA. This digestion mixture was incubated at 37 Celsius for 2 hours in a thermocycler and then introduced to 70 vol% glycerol in water until the mixture was 80 vol% digestion material and loaded into a 1.2 m/v% agarose in 1x TAE gel containing 0.0185 vol% SYBR® Safe DNA Gel Stain 10000x Concentrate with a ≤15% 2 log DNA ladder in 70% glycerol loaded well for accurate size comparison. With 1x TAE as the electrophoresis buffer, the gel was run at 110 V for 1.5 hours and imaged using a blue light imager. When the appropriate band size was confirmed, the 1071 bp band was excised from the gel using a sterile scalpel and extracted from the gel using a GenElute™ Gel Extraction Kit. The concentration was found through a Nanodrop 2000c Spectrophotometer using DI water as the blank with a baseline correction of 340 nm and then frozen at -20 Celsius for later use.

To create the double-stranded insert for the PsbA promotor and ribosome binding site (RBS), 2 sequences were purchased from Integrated DNA Technologies® (IDT): EcoR1_PsbA_RBS_Xba1_1 and EcoR1_PsbA_RBS_Xba1_2. These two 113 base sequences of DNA contain the genetic material for an active promoting sequence to influence *Rhodococcus opacus* PD630's proteins to attach to the plasmid and then proceed to transcribe and translate the sequence to make the protein that the efe gene encodes for. To anneal these sequences together to become a viable insert for the plasmid, we mixed an equimolar solution of both sequences into a small PCR vial and heated the vial in a dry bath to 98 Celsius. The mixture was then heated for 10 minutes before being removed from the dry bath and allowed to cool to room temperature slowly. Containing the double-stranded piece of DNA, the concentration of the mixture was found through a Nanodrop 2000c Spectrophotometer with a baseline correction of 340 nm with a DI water blank solution and frozen at -20 Celsius for later use in cloning.

The pBAV1K backbone containing the kanamycin resistance required for the selection of transformant bacteria was harvested from the digestion of the known pBAV1K-T5-gfp plasmid contained in bought genetically modified *Escherichia coli* stocks. To extract the backbone and purify the DNA fragment required for cloning, sterile technique was followed to inoculate LB-Kan plates with genetically modified *Escherichia coli* containing pBAV1K-T5-gfp and grown in a non-rotating incubator at 37 Celsius overnight or until colonies were large enough to be harvested through a sterilized and flamed inoculation loop. Then, colonies were harvested and introduced in 2 mL of 50 µg Kanamycin/ mL LB tubes and incubated at 37 Celsius and 150 rpm overnight. These resultant cells were harvested and pBAV1K-T5-gfp was extracted using a GenElute™ Plasmid Miniprep Kit with DI water as the final elute. Post extraction, the plasmid was stored at -20 Celsius until used in a 50 µL digestion composed of 5 µL of 10x rCutsmart buffer, 1 µL of EcoRI-HF restriction enzyme, 1 µL of SpeI-HF restriction enzyme, and 43 µL of plasmid DNA. This digestion mixture was incubated at 37 Celsius for 2 hours in a thermocycler and then introduced to 70 vol% glycerol in water until the mixture was 80 vol% digestion material and loaded into a 1.2 m/v% agarose in 1x TAE gel containing 0.0185 vol% SYBR® Safe DNA Gel Stain 10000x Concentrate with a ≤15% 2 log DNA ladder in 70% glycerol loaded well for accurate size comparison. With 1x TAE as the electrophoresis buffer, the gel was run at 110 V for 1.5 hours and imaged using a blue light imager. When the appropriate band size was confirmed, the 2811 bp band was excised from the gel using a sterile scalpel and extracted from the gel using a GenElute™ Gel Extraction Kit. The concentration was found through a Nanodrop 2000c spectrophotometer using DI water as the blank with a baseline correction of 340 nm and then frozen at -20 Celsius for later use in cloning the final engineered plasmid called pBAV1K-PsbA-efe.

Cloning Experiment Procedure and Conditions for Success

Three experiments were conducted to process the plasmid components and synthesize the final engineered plasmid. All of these experiments ran through the same procedure but varied in 3 levels of composition: a “Big” concentration, a “High” concentration of plasmid components, and a “Low” concentrated reaction for the components. The “Big” concentrated reaction followed the detailed assembly protocol for a 4-6 fragment assembly in a NEBuilder® HiFi DNA Assembly Cloning Kit, where the vector to insert ratio is 1:1 in pmol and the total amount of fragments was 0.5 pmol with 0.01 µL PsbA (0.2 pmol), 0.705 µL of efe gene (0.2 pmol), and 30.3 µL of pBAV1K backbone (0.2 pmol). Then, 31 µL of NEBuilder HiFi DNA Assembly MasterMix was added. Both of the “High” and “Low” concentrated reactions followed the detailed assembly protocol for a 2-3 fragment assembly in a NEBuilder® HiFi DNA Assembly Cloning Kit, where the vector to insert ratio is 1:2 in pmol and the total amount of fragments is varied on achievable pipette volume. In the “High” reaction, 1 µL of the backbone (27 pmol), 1.1 µL of PsbA solution (54 pmol), and 1.9 µL of efe gene (54 pmol) were combined with 10 µL of NEBuilder® HiFi DNA Assembly MasterMix was added, and the solution was brought to 20 µL by DI Water, resulting in 135 pmol in the total solution. Comparatively, the “Low” reaction was composed of 0.1 µL of the backbone (2.7 pmol), 0.11 µL of PsbA solution (5.4 pmol), and 0.19 µL of efe gene (5.4 pmol) combined with 10 µL of NEBuilder® HiFi DNA Assembly MasterMix, and the solution was brought to 20 µL by DI water, resulting in 13.5 pmol of components in the total solution. All assembly reactions were then placed into a thermocycler at 50 Celsius for 60 minutes and then stored on ice or frozen at -20 Celsius until used to transform

provided chemically competent *Escherichia coli* cells through the NEBuilder® Competent *E. coli* sampler.

To transform the provided competent cells, the 2 tubes of chemically competent cells were thawed on ice and one tube was introduced to 2 µL of the chilled assembly reaction, gently pipetting the reaction mixture throughout the solution. Both tubes were then left on ice for 30 minutes and then heat shocked at 42 Celsius in a dry bath for 30 seconds. Post-shock, the samples were transferred back to the ice for another 2 minutes, removed from the ice, and 950 µL of room temperature SOC media was added to the control and experimental cell tubes. Both cell tubes were then incubated at 37 Celsius and 250 rpm for 60 minutes while 2 LB-Kan plates were warmed in a 37 Celsius stationary incubator. After incubation, the tubes were spun down at 10000 rpm for 4 minutes, the supernatant was discarded, and the cells were resuspended in 100 µL of SOC media. The 100 µL of resuspension solution was then spread onto the warmed plated, allowed to dry, and incubated at 37 Celsius overnight or until colonies first appear on the plates. A successful inoculation was achieved when more than 3 colonies were visibly seen on the experiment plate, no colonies appeared on the control plate, and the extracted plasmid from the resulting colonies could be accurately identified as pBAV1K-PsbA-efe through EcoRI-HF-SpeI-HF and NdeI digestion.

All test digestions for the cloned colonies were run at 37 Celsius for 2 hours. The EcoRI-HF-SpeI-HF digest was composed of 8 µL test plasmid, 1 µL of 10x rCutsmart Buffer, 0.5 µL of EcoRI-HF digestive enzyme, and 0.5 µL of SpeI-HF digestive enzyme. NdeI digests were composed of 8.5 µL test plasmid, 1 µL of 10x rCutsmart Buffer, and 0.5 µL of NdeI digestive enzyme. Post incubation, the samples were combined with 3 µL of autoclaved 70 vol% glycerol in DI Water and loaded into a 1.2 m/v% agarose in 1x TAE gel containing 0.0185 vol% SYBR® Safe DNA Gel Stain 10000x Concentrate with a ≤15% 2 log DNA ladder in 70% glycerol (13 µL in total ladder solution) loaded well for accurate size comparison. With 1x TAE as the electrophoresis buffer, the gel was run at 110 V for 1.5 hours and imaged using a GelDoc™ Imager. The image was then enhanced to show all bands present and then printed to accurately evaluate the size of all bands present as well as remarking any missing bands in writing. All digestions were also performed twice to remove any possible error in digestion or imaging.

Electroporation Procedure and Conditions for Success

Two main procedures were attempted in trying to establish competent *Rhodococcus opacus* PD630 cells, one defined in Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application of biosynthesis of poly (3-hydroxyalkanoic acids) by Kalscheuer et al. in 1999 and a modified version of the same protocol established in the article Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases by Huang et al. in 2015. The main difference in technique results from the supplemented growth media used to produce the competent cells and the regenerative media used post-electroporation. In Kalscheuer et al.'s article, they define an electroporation technique that uses Nutrient Broth media supplemented with 1 m/v% sucrose and 0.85 m/v % glycine as its main growth media and pure Nutrient Broth as its regenerative media. On the other hand, Huang et al. detail a procedure in their aforementioned article that uses Luria-Bertani Broth supplemented with 1 m/v% sucrose and 0.85% glycine as its main growth media and pure SOC

media as its post-electroporation regenerative media. Beyond those differences, the procedures follow the same technique detailed in the following paragraph that was replicated to produce genetically modified, competent *Rhodococcus opacus* PD630.

To make the competent cells, 2 mL of Nutrient Broth (NB) media or LB Broth was inoculated with 1 loopful of wildtype *Rhodococcus opacus* PD630 using a sterilized and flamed inoculation loop, allowing to grow overnight in a shaking incubator at 30 Celsius and 200 rpm for Kalscheuer et al.'s procedure and 28 Celsius and 200 rpm for 24 hours for Huang et al.'s procedure. Then, 56 mL of growth media was supplemented with 0.58 g sucrose and 0.493 g glycine in a 500 mL Erlenmeyer flask, swirled until homogenous, and the overnight/24-hour culture was added into the flask. The Erlenmeyer flask was then labeled and placed into a shaking incubator at 30 Celsius and 105 rpm until an OD of 0.5 could be achieved using a spectrophotometer with a baseline correction at 600 nm in 10 mm UV cuvettes and a blank solution composed of uninoculated supplemented growth media. 50 mL of the final growth media were harvested into a 50 mL capped conical tube where it would be centrifuged at 2200x g for 10 minutes at 4 Celsius and, on ice, the supernatant was removed. The cells were resuspended in 10 mL of ice-cold DI water by gentle vortexing on ice and then centrifuged again under the same conditions, washing the cells. The resulting supernatant was removed, and the process was repeated for a final time. After washing for the second time, the cells were concentrated 20-fold in ice-cold DI water on ice and then combined with autoclaved room temperature glycerol until the solution was 10% glycerol by volume. It is here where the two methods differ again as Kalscheuer et al.'s NB-based competent cells are stored in 800 µL aliquots at -80 Celsius while Huang et al.'s LB-based competent cells are stored in 400 µL aliquots at -80 Celsius.

Electroporation for both procedures follows similar paths as in the NB-based procedure, an experimental cell volume of 400 µL competent *Rhodococcus opacus* PD630 cells were mixed with pBAV1K-T5-gfp DNA until a final concentration of 0.1 µg/mL was achieved. Both the control and experimental cell volumes were placed into 2mm electrocuvettes, where they were incubated at 40 Celsius in a dry bath for 5 minutes and then removed to be pulsed at an electroporator with the following conditions: 600 Ω, 2000 V, and 25 µF. Then in Kalscheuer et al.'s procedure, the cells are immediately diluted with 600 µL of non-supplemented NB and allowed to regenerate at 30 Celsius for 4 hours. In Huang et al.'s procedure, an experimental cell volume of 200 µL competent *Rhodococcus opacus* PD630 cells was mixed with pBAV1K-T5-gfp DNA until a final concentration of 0.1 µg/mL was achieved. Both the control and experimental cell volumes were placed into 2mm electrocuvettes, where they were incubated at 40 Celsius in a dry bath for 5 minutes, placed on ice for 10 minutes, and then removed to be pulsed at an electroporator with the following conditions: 600 Ω, 2000 V, and 25 µF. Then in the LB-based procedure, the cells are immediately diluted with 800 µL of non-supplemented SOC media and allowed to regenerate at 28 Celsius for 4 hours. After 4 hours, the control and experimental tubes in both procedures were spun down at 10000 rpm for 4 minutes, resuspended in 200 µL of SOC or NB media, and plated on two warmed, labeled LB-Kan plates. The plates were allowed to absorb the liquid media and incubated in a stationary incubator at 30 Celsius until colonies arrived on one of the two plates. Success in electroporation was characterized by the presence of 3 or more colonies on the experimental cell plate and no colonies present on the

control plate. All experiments with both the NB and the LB-based electroporation techniques were performed 3 times to remove data that resulted from random chance.

Results

Growth Media Study

To see how *Rhodococcus opacus* PD630 grows in both Luria-Bertani Broth and synthetic human urine, growth studies with 3 labeled flasks of each media were conducted. These flasks were grown at 30 Celsius and 105 rpm until 3 values of close to stationary values were achieved, where the contents of the flasks were discarded through the introduction of bleach, washed thoroughly, and then autoclaved for insured sterility. Optical density values for these growth curves were recorded around every 6 hours and placed into the scatter plots shown in figures 1 and 2. Within both of these graphs, the x-axis depicts the time that has passed since the first inoculation of the Erlenmeyer flask growth media while the y-axis represents the optical density values that were measured in a 10 mm cuvette in a Nanodrop 2000c Spectrophotometer compared to an uninoculated growth media. As expected, the bacteria after initial inoculation suffered a minor dip in optical density and then after becoming accustomed to their environment, the bacteria started to grow in the exponential growth phase, doubling at a consistent rate. After a couple of days, the bacteria's growth began to slow until approaching the stationary phase. While the overall curvature of the graphs seen in figures 1 and 2 are nearly identical, there are key differences worth noting between *Rhodococcus opacus* PD630's growth in LB broth in comparison to its growth within synthetic human urine.

When growing in synthetic human urine, the peak optical density within the stationary phase was significantly lower than that in Luria-Bertani broth, achieving an average maximum optical density of 1.297±0.135 for the urea-rich media compared to an average maximum optical density of 6.074±0.597 for Luria-Bertani broth. These results, while expected, suggest that in a less nutrient-dense media like the synthetic human urine, *Rhodococcus opacus* PD630 will achieve a lower cell-to-volume ratio, causing a lower overall optical density. Additionally, it suggests that in a complete media like Luria-Bertani broth, *Rhodococcus opacus* PD630 enters the exponential growth phase and doubles at a higher rate when compared to the growth observed in synthetic human urine. With these results in mind, it can be concluded that the genetically modified *Rhodococcus opacus* PD630 will likely grow in a lower cell density in the synthetic human urine compared to its potential density in Luria-Bertani broth, which could impact the amount of ethylene that could be harvested from the cell culture as a whole.

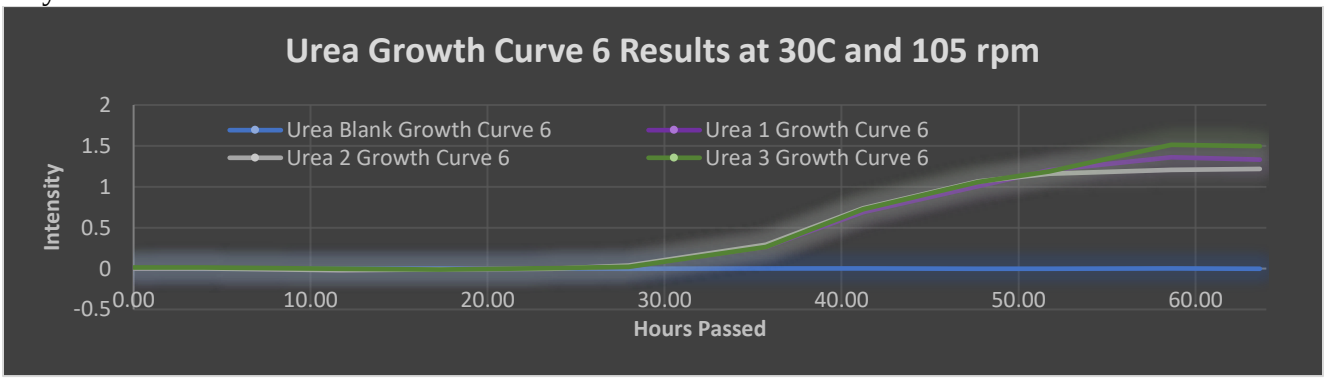

Figure 1: A scatter plot depicting the optical density values for growth flask samples 1, 2, and 3 when R. opacus PD630 is allowed to progress through its growth cycle up to the stationary phase compared to a control uninoculated synthetic human urine sample. The average stationary phase optical density for this particular round of growth curve studies was 1.297. Data collection terminated after 105.7 hours, where the optical density readings differed too highly from the main trend to continue.

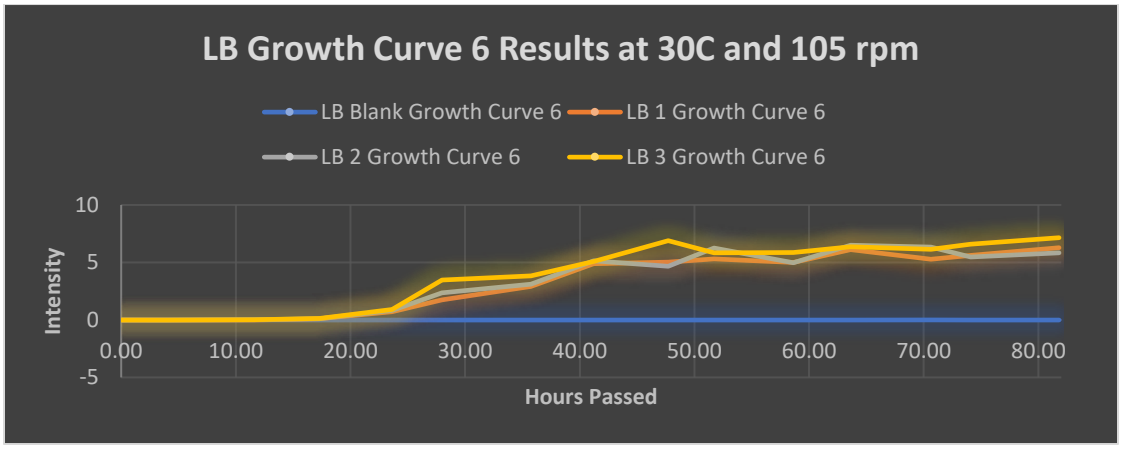

Figure 2: A scatter plot depicting the optical density values for growth flask samples 1, 2, and 3 when R. opacus PD630 is allowed to progress through its growth cycle up to the stationary phase compared to a control uninoculated Luria-Bertani Broth sample. The average stationary phase optical density for this particular round of growth curve studies was 6.074, found through a 1:10 dilution of media. Data collection terminated after 105.7 hours, where the optical density readings differed too highly from the main trend to continue where they would appear significantly higher and then drop to the main trend within hours of the previous data point.

Plasmid Component Isolation and Cloning

Post extraction of the efe gene from *Escherichia coli* vectors, 10 µL of each sample vial (a small PCR vial labeled efe, concentrated fresh efe gene, and fresh efe gene extracted from a glycerol stock colony) was place into PCR vials and mixed evenly with 3 µL of 70 vol% glycerol in DI water. Then, as seen in the leftmost UV image in figure 3, the samples were loaded from the bottom of the gel to the top in the following order with digestions of original pBAV1K-T5-gfp using EcoRI-HF and SpeI-HF restriction enzymes: 2 log DNA ladder (3:10 ladder to 70% glycerol ratio), a blank well, EcoRI-HF-SpeI-HF digest of pBAV1K-T5-gfp vial 1, EcoRI-HF-SpeI-HF digest of pBAV1K-T5-gfp vial 2, another blank well, a small PCR vial labeled efe, concentrated fresh efe gene, and the fresh efe gene extracted from a glycerol stock colony. Based on the resultant image produced by a GelDoc™ EZ Imager after running the electrophoresis gel at 110V for 1.5 hours, only the concentrated fresh efe gene and the fresh efe gene extracted from a glycerol stock colony could produce the correct 1071 band without any other DNA bands present in the sample. The small PCR vial was proven to be a discardable sample as it appears to be contaminated with multiple DNA fragments of regulated sizes similar to a commercial DNA ladder. Both fresh samples were used in the cloning experiments conducted.

After combining multiple vials of pBAV1K-T5-gfp and running a 50 µL digestion reaction with restrictive enzymes EcoRI-HF and SpeI-HF, 8.3 µL of 70 vol% glycerol in water was vortexed into the digestion reaction and loaded in a well with diluted 2 log DNA ladder (in a 2:10 ratio of ladder to 70% glycerol) with a low concentration digestion of pBAV1K-T5-gfp with EcoRI-HF and SpeI-HF as shown in the middle image of figure 3. When a high concentration band around

2811 bp was observed in the gel, it was carefully excised and purified with a GenElute Gel Extraction Kit. Since the condensed vial originally resulted in the correct band size, it was used in the cloning experiments to produce the final cloning plasmid pBAV1K-PsbA-efe.
When the “Low”, “High”, and “Big” concentration cloning procedures were followed and the plates were allowed to grow, there were significant differences in the number of colonies grown on the plates, but the EcoRI-HF-SpeI-HF and the NdeI restriction enzyme digests resulted in the same digestion pattern of our proposed pBAV1K-PsbA-efe plasmid observed across all colonies. Since the spun down plates should have the highest concentration of cells in a small volume, they were used to represent the overall effectiveness of the cloning procedure tested as the number of colonies observed would only result from complete plasmids. The “Low” concentration cloning formula spun plate only resulted in one observable small colony while the “High” concentration cloning formula resulted in small, scattered colonies throughout the whole plate. Most of all, the “Big” concentration cloning reaction resulted in large colonies scattered over the entire plate, providing the most promise in having highly concentrated pBAV1K-PsbA-efe vials after the GenElute Plasmid Miniprep Kit for viewable digestion bands.
Once the proposed pBAV1K-PsbA-efe was extracted, it was digested in a 10 µL digestion reaction with EcoRI-HF, SpeI-HF, and NdeI at 37 Celsius for 2 hours in 10x rCutsmart buffer. The digestion reaction of 3 colonies, referred to as colony Rho, colony Sigma, and colony Tau, were mixed with 3 µL of 70% glycerol in DI water and loaded into a 1.2% agarose gel in 1x TAE running buffer in the following order in Figure 3’s right-most UV image from the bottom to the top well: 2 log DNA ladder, blank, NdeI digest of pBAV1K-PsbA-efe colony Rho, EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Rho, blank, NdeI digest of pBAV1K-PsbA-efe colony Sigma, EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Sigma, blank, NdeI digest of pBAV1K-PsbA-efe colony Tau, and the EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Tau. In each EcoRI-HF-SpeI-HF reaction, a DNA fluorescent band appeared between the 2 kb and 3kb ladder bands, but no evidence of the insert could be found in the later part of the gel. Additionally, the NdeI restriction enzyme didn’t appear to cut the proposed plasmid, but the proposed uncut plasmid is around the correct size at approximately 3900 bp. From these results, we cannot confirm the cloning procedures produced the right engineered plasmid within during the cloning reactions or if the bacterial vectors modified the DNA to remove specific sequences from the proposed plasmid’s complete nucleotide sequence.

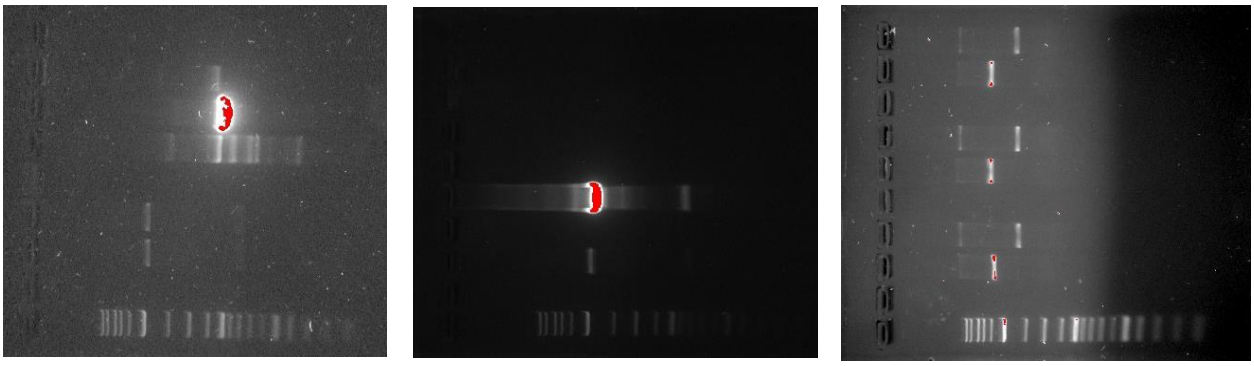

Figure 3, Left: A UV image produced by a GelDoc™ EZ Imager of multiple efe gene vials for potential use in cloning. The wells depicted to the left of this image are the following from the bottom-most well to the top: 2 log DNA ladder, blank, EcoRI-HF-SpeI-HF digest of pBAV1K-T5-gfp vial 1, EcoRI-HF-SpeI-HF digest of pBAV1K-T5-gfp vial 2, blank, a small PCR vial labeled efe, concentrated fresh efe gene, and fresh efe gene extracted from a glycerol stock colony. The gel was performed to test the most viable vial of efe gene to be used for cloning and to source colonies that can be harvested for efe gene in further experiments.

Figure 3, Middle: A UV image produced by a GelDoc™ EZ Imager depicting the pBAV1K backbone before gel extraction. Proceeding after a high concentration of DNA digested by EcoRI-HF and SpeI-HF digestive enzymes, a 1.2 m/v% agarose gel was run at 110 V for 1.5 hours until these bands were visible to the imager and the bands were isolated enough for gel extraction. The wells depicted to the left of this image are the following from the bottom-most well to the top: 2 log DNA ladder, blank, an uncondensed vial of pBAV1K-T5-gfp digestion of EcoRI-HF and SpeI-HF restrictive enzymes, blank, and a highly condensed vial of pBAV1K-T5-gfp digestion by EcoRI-HF and SpeI-HF restrictive enzymes. The bold 2811 bp fragment was then excised from the gel and purified to be used in cloning for the engineered pBAV1K-PsbA-efe plasmid.
Figure 3, Right: A UV image produced by a GelDoc™ EZ Imager of the resulting digestion bands of the cloned pBAV1K-PsbA-efe plasmid with EcoRI-HF, SpeI-HF, and NdeI restrictive enzymes. The wells depicted to the left of this image are the following from the bottom-most well to the top: 2 log DNA ladder, blank, NdeI digest of pBAV1K-PsbA-efe colony Rho, EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Rho, blank, NdeI digest of pBAV1K-PsbA-efe colony Sigma, EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Sigma, blank, NdeI digest of pBAV1K-PsbA-efe colony Tau, and EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe colony Tau. Based on these results, restrictive enzyme NdeI did not cut any samples and the smaller bands are too low of a concentration to be seen for the EcoRI-HF-SpeI-HF digest of pBAV1K-PsbA-efe, resulting in failed identification of the final product.

Electroporation

After being pulsed, the bacteria resultant from following Kalscheuer et al.'s electroporation procedure produced time constants ranging from 8 to 12.7 ms for the experimental bacterial samples and 10 -12.7 ms for the control samples, nearly doubling the 3-5 ms values presented in Establishment of a gene transfer system for *Rhodococcus opacus PD630* based on electroporation and its application of biosynthesis of poly (3-hydroxyalkanoic acids). These samples were then spread onto plates and did not show colonies on the experimental plate until 9 to 11 days after inoculating the LB-Kan plates. On the 9th day, only 2 colonies were present on the surface of the agar on the experimental plates, failing to meet the criteria set for successful electroporation of *Rhodococcus opacus* PD630, even after taking nearly 3 times as long as Kalscheuer et al.'s proposed transformant time, 3-4 days. Due to poor camera quality at the time recording the colonies, images were not provided for this documentation.

Meanwhile, the *Rhodococcus opacus* PD630 samples following Huang et al.'s electroporation technique with LB broth as its growing medium produced successful results. Even though the time constants reported from pulsing the bacteria were 2 times the time constants reported in the article Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases (12.3 and 12.1 ms for the control and the experimental samples respectively in comparison to the reported 6 ms), transformant colonies were observed in a shorter amount of time compared to the plates from the nutrient broth-based plates, producing colonies after 5 days. For the initial test of the LB broth procedure, 7 small colonies were present on day 5, where they were pulled from the incubator, photographed, recorded in writing, and then wrapped in Parafilm to be stored at 4 Celsius until it can be tested for plasmid identification and evaluated for the integrity of the plasmid through the transformation process. This process was then repeated 3 times to remove any results due to random chance, averaging in a colony observation time averaging 5.25 days with time constants of 12.2 ± 0.13 ms for the control sample and 12.4 ± 0.53 ms for the experimental sample. 1 in 4 experiments with the LB procedure performed overall had failed due to contamination in the control plate, suggesting preventive measures against contamination to be put into place. The other 3 trials produced experiment plates with 3-7 colonies of kanamycin-resistant *R. opacus* PD360 without observable bacteria growth on their respective control plates, as seen in Figure 4. Exact identification of the

transformed plasmid composition within the transformed colonies are in progress, as the colonies resulting from the electroporation are too small to produce observable bands in electrophoration gel imaging.

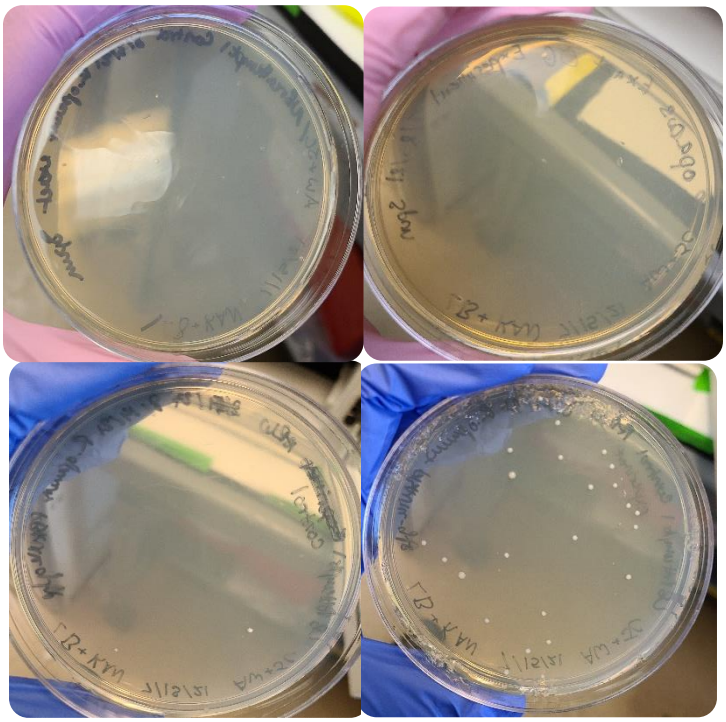

Figure 4: As seen to the top left and right of the image, the control and experimental plates from one of the runs of the nutrient broth based electroporation procedure. While the control plate meets the passing criteria of having no resulting growth when the pBAV1K-gfp plasmid is not present, the experimental plate shows little to no growth, resulting in a failure of the experiment. This led to producing electroporated cells using Huang et al.'s method established in 2015 that uses Luria-Bertani Broth and is main medium and SOC medium as its regenerative feed stock. As this led to more growth, as seen to the bottom right of this image, it was also more susceptible to contamination of the SOC media that would then be observed on the control plate, seen to the bottom left. Further experimentation with extra sterile precision would need to be preformed later on to ensure that once pBAV1K-efe is made and correctly identified it can enter the cells without a false positive or negative reading.

Discussion

In this report, we had designed several experiments to demonstrate *Rhodococcus opacus* PD630's ability to grow on synthetic human urine to replicate the bacteria's response to sterile astronaut urine based on a procedure presented in an article called A New Artificial Urine Protocol to Better Imitate Human Urine by Sarigul et al. In addition, we designed experiments on electroporation methods for the *Rhodococcus opacus* PD630 cells and cloning a plasmid that theoretically would be capable for converting fatty acid precursor molecules to ethylene that would, in turn, lay the foundation for future ethylene detection experiments on the genetically modified *Rhodococcus opacus* PD630 via gas chromatography. Within these experiments, it was discovered *Rhodococcus opacus* PD630 grew to a lower optical density in synthetic human urine when compared to a complete nutrient medium like Luria-Bertani broth. Additionally, a significantly large pmol concentration of cloning reaction to synthesize an engineered plasmid proved to produce the highest number and largest transformant colonies, despite not being able to confirm the identity of the plasmid post-transformation and extraction. Lastly, it was found

that utilizing supplemented Luria-Bertani broth as a growth medium and SOC media as a regenerative media for electroporation provided the largest amount of transformant *Rhodococcus opacus* PD 630 when compared to a similar procedure using nutrient broth. Despite these conclusions, there is room for improvement in how these experiments were conducted to enhance the experiments and produce enhanced results.

Within the growth study experiments, more measurements could be taken throughout the day while increasing sterile technique practices by only accessing the growth flasks and blank media within a laminar flow hood. These changes would provide more insight into the rate at which the bacteria grows in both Luria-Bertani broth and synthetic human urine by having more data points as well as ensuring that the same blank media can be used throughout the experiment without the risk of contamination. As for the other experiments, all DNA extracted could be concentrated to a high overall concentration to save material and reduce the need to constantly shear more colonies for more pBAV1K-T5-gfp for control testing and backbone extraction. Additionally, more care in extracting the backbone from gels could be implemented to ensure the purity of the backbone cloning component, which could lead to correct transformation and cloning reactions in competent *Escherichia coli* cells containing synthesized pBAV1K-PsbA-efe plasmid. Once the pBAV1K-PsbA-efe plasmid is identified through correct band lengths in UV imaging from the digestion of NdeI, EcoRI-HF, and SpeI-HF restriction enzymes, the plasmid can be transformed into competent *Rhodococcus opacus* PD630 by following the procedure detailed in the article Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases, later to extracted again to be digested for confirmation of plasmid identity and integrity through the electroporation process.

Progressing forward, the next steps would be to introduce a complete, identified pBAV1K-PsbA-efe plasmid into competent *Rhodococcus opacus* PD630 cells to have a renewable source of the plasmid. Then, growth studies in synthetic human urine and Luria-Bertani broth would be performed to see if the introduction of the plasmid influences the growth pattern of the bacteria compared to the wild-type *Rhodococcus opacus* PD630. These growth studies would be very similar to the ones previously performed on the wild type bacteria, recording the optical density in the growths 3 to 4 times a day around every 6 hours to determine the effect the plasmid has on bacterial growth, terminating the experiment when 3 consecutive points of a similar numeric value (<5% change) are recorded for one of the 3 experimental vials. These new growth curves will differ in the standardized growth media composition as the growth media the bacteria will be growing in will now contain kanamycin in a 50 µg/mL concentration to select for the transformed cells and kill any contaminant wild type cells. It is predicted through this change in growth media conditions, the transformed cells will accomplish a lower optical density in both the synthetic human urine and Luria-Bertani broth since there will be fewer initial cells surviving after the initial inoculation and the unknown effect of ethylene production if it occurs, will have on the cellular growth of *Rhodococcus opacus* PD630. Once the growth studies are completed and the plasmid is identified, experiments centered around gas chromatography detection of ethylene would be performed to access the plasmid's success in converting precursor fatty acids to ethylene particles. Using ethylene as the calibration gas for an Agilent Technologies 7890B GC System, we would carefully run samples of grown *Rhodococcus opacus* PD630 inoculated synthetic human urine media through the gas chromatographer and observe the graph created for peaks at the location of the calibration peaks in the literature to our own from running the

sample. If any intensity peak in the ethylene region of the graph is present, a positive result will be recorded and if no peak is present, the sample would be confirmed to be negative for ethylene production. If all samples test negative for ethylene detection, different aspects of the project could be modified to produce ethylene with the plasmid including but not limited to changing the conditions in which the ethylene is detected like the temperature of the machine, modifying components of the engineered plasmid for a more active promoter or different ethylene conversion enzyme, providing more growth time for the transformed bacteria, or changing the strain of *Rhodococcus opacus* used to B4 instead of PD630, which has been suggested to behave similarly to *Rhodococcus opacus* PD630 metabolically in literature (Castro et al. 2016). These changes will provide other avenues to explore and may influence the results towards a positive outcome.

Furthermore, when this research is completed and *R. opacus* PD630 can produce ethylene from the efe protein and fatty acid catabolism precursor molecules, research into introducing the gene through more biologically cumbersome means can be established to integrate the efe gene into the bacterial genome. If in the genome, the efe gene would not risk being kicked out from its host cell in plasmid form and can be part of the overall transcription and translation process of *R. opacus* PD630's circular chromosome. To perform this feat of genetic engineering, two potential avenues could take place: modifying a bacteriophage that is selective for our strain to make it unable to reproduce but spread the efe gene through transduction or to implement usage of CRISPR-Cas-9 to insert the gene physically into the circular chromosome our strain and deleting deleterious or accessory DNA in the cell to make room for the efe gene, our promoter, the RBS, and a termination gene. While these avenues would take a lot longer to preform and even longer to clarify through mapping *Rhodococcus opacus* PD 630's genome, this would result in permanence of the efe gene strain that could be taken to space with a smaller volume of initial kanamycin needed on the voyage and during colonization as the strain would need to be screened for wild-type bacteria every now and then but not as often as a strain with only our original pBAV1K-efe plasmid.

Literature Cited

Agilent Technologies. *Agilent 7890B Gas Chromatograph Operation Manual.* https://www.agilent.com/cs/library/usermanuals/Public/7890B_Operation.pdf.

Castro, A., Rocha, I., Alves, M., & Pereira, M. (2016). *Rhodococcus opacus* B4: a promising bacterium for production of biofuels and biobased chemicals. *AMB Express*, *6*(1):35. https://doi.org/10.1186/s13568-0160207-y.

Huang, L., Zhao, L., Zan, X., Song, Y., & Ratledge, C. (2015). Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases. *Biotechnology Letters, 38*(6). 999-1008. https://doi.org/10.1007/5101529-016-2072-9.

Kalscheuer, R., Arenskötter, M., & Steinbüchel, A. (1999). Establishment of a gene transfer system for *Rhodococcus opacus PD630* based on electroporation and its application of biosynthesis of poly (3-hydroxyalkanoic acids). *Applied Microbiology and Biotechnology*, *52*(4). 508-515. https://doi.org/10.1007/s002530051553.

Lomond, J., & Tong, A. (2011). Rapid Analysis of Dissolved Methane, Ethylene, Acetylene, and Ethene using Partition Coefficients and Headspace- Gas Chromatography. *Journal of Chromatographic Science*, *49*(6). 469-475. https://doi.org/10.1093/chrsci/49.6.469.

Na, K., Nagayasu, K., Kuroda, A., Takiguchi, N., Ikeda, T., Ohtake, H., & Kato, J. (2005). Development of a Genetic Transformation System for Benzene-Tolerant *Rhodococcus opacus* strains. *Journal of Bioscience and Bioengineering*, *99*(4). 408-414. https://doi.org/10.1263/jbb.99.408.

NASA (2008). *Advanced Space Transportation Program: Paving the Highway to Space*. https://www.nasa.gov/centers/marshall/news/background/facts/astp.html.

Ouchane, S., Picaud, M., Reiss-Husson, F., Vernotte, C., & Astier, C. (1996). Development of gene transfer methods for *Rubrivivax gelatinosus* S1: construction, characterization and complementation of a *puf* operon deletion strain. *Molecular and General Genetics MGG*, *252*. 379-385. https://doi.org/10.1007/BF02173002.

Sarigul, N., Korkmaz, F., & Kurultak, İ. (2019). A New Artificial Urine Protocol to Better Imitate Human Urine. *Scientific Reports*, *9.* 1-11. https://doi.org/10.1038/s41598-019-56693-4.

Sekizaki, T., Tanoue, T., Osaki, M., Shimoji, Y., Tsubaki, S., & Takai, S. (1998). Improved Electroporation of *Rhodococcus equi*. *The Journal of Veterinary Medical Science, 60* (2). 277-279. https://doi.org/10.1292/jvms.60.277.