Ethylene Synthesis by Genetically Modified *Rhodococcus opacus* PD630 on Synthetic Human Urine

Fatty Acid Accumulation and Utilization through *Rhodococcus opacus'* response to Synthetic Human Urine

Presentation Overview

- Goal of the Project and *Rhodococcus opacus'* role
- Evaluating Growth of *R. opacus* on Synthetic Urine vs. LB Broth
- Competent Cell Synthesis and Electroporation
- Cloning Plasmid Synthesis Procedure and Identification
- Growth Studies Past Plasmid Introduction
- Ethylene Detection via Gas Chromatography
- Closing Statements

Goal Establishment and Control Microbial Growth

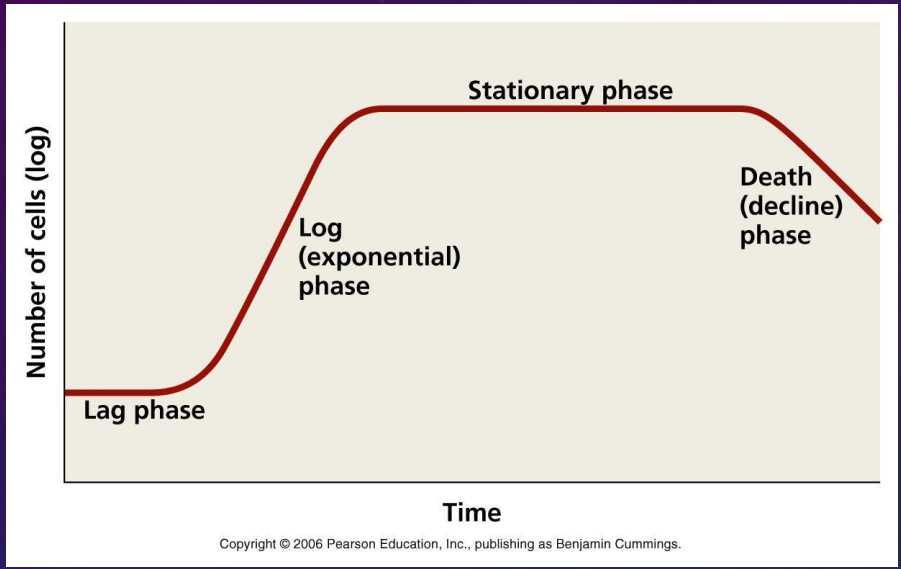
Goal of the Project

- **Modifying a bacterium to produce ethylene from genetic modification to accommodate NASA's Center for the Utilization of Biological Engineering in Space.**
- **Modeled with *Rhodococcus opacus PD630***
  - **Metabolically diverse enough to eat urea, a compound largely found in human urine.**
  - **Continues to grow at the same time as it is accumulating fatty acids which can be converted to ethylene with a protein.**
- **Ethylene produced from waste could be used to decrease price of colonization through providing material for equipment synthesis.**

Development of Synthetic Human Urine

- Followed a detailed procedure proposed by Sarigul et al. in their publication A New Artificial Urine Protocol to Better Imitate Human Urine.
- Shares characteristics to real human urine, a common waste product in space travel.
- Contains plenty of Urea, a component the *Rhodococcus opacus PD630* can utilize as a carbon source.
- High salinity to promote the production of fatty acid precursors such as TAG that can later be converted to ethylene through an efe gene in a plasmid.

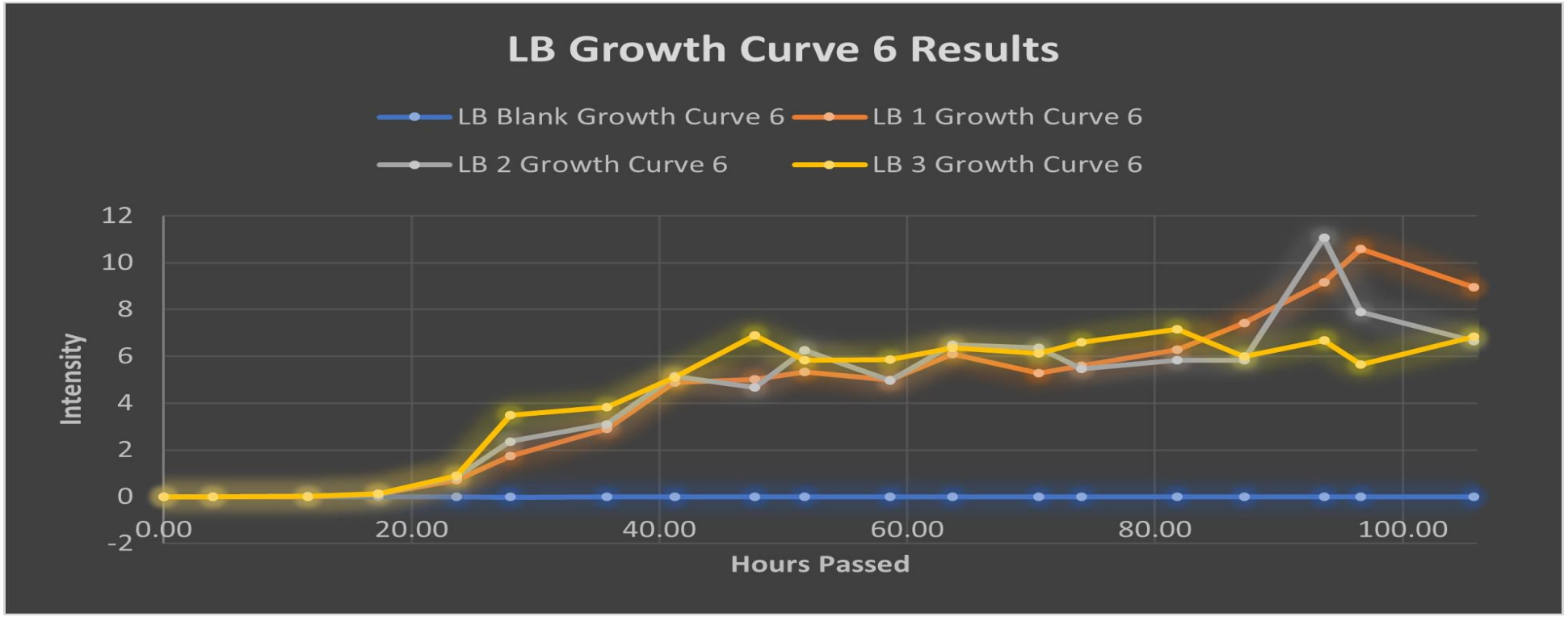
Growth Conditions & Criteria for Entry of Stationary Phase



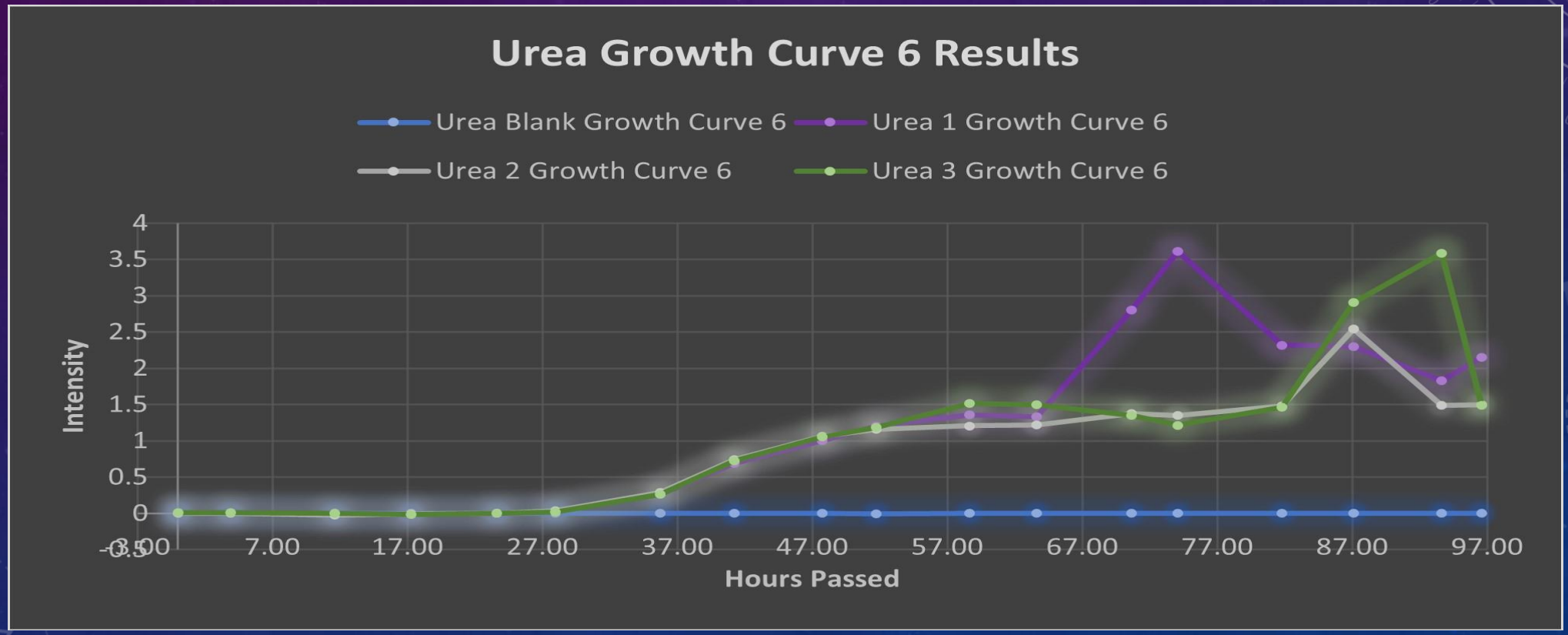
- Beakers and inoculation procedure
  - 30 C 150 rpm in 500 mL flasks with 90 mL of growth media
  - Seed solution conditions: 6mL with 3 loops, 30 C 150 rpm for 8 hours
- Recording of data protocol
  - Uses spectrophotometer
  - Every 6 hours, 600nm baseline wavelength, cuvette measurements with 1 mL. Dilute as needed to remain in 1.5 OD manufacture limit and calculate true optical density
- Conditions for termination
  - 3 points of OD that did not vary more than 5% from an average or a sudden spike after 2 points of a steadying value and then a rapid drop back to 5% of the previous steady value (or 5% within its value) in irregular points (up down up down up down was considered as noise and cause for termination). Consistent for approximately 18 hours is the short version of termination.

https://www.quora.com/What-is-bacterial-growth-What-are-the-different-phases-of-a-bacterial-growth-curve

Established Luria Broth Growth Curve with *Rhodococcus opacus PD 630*

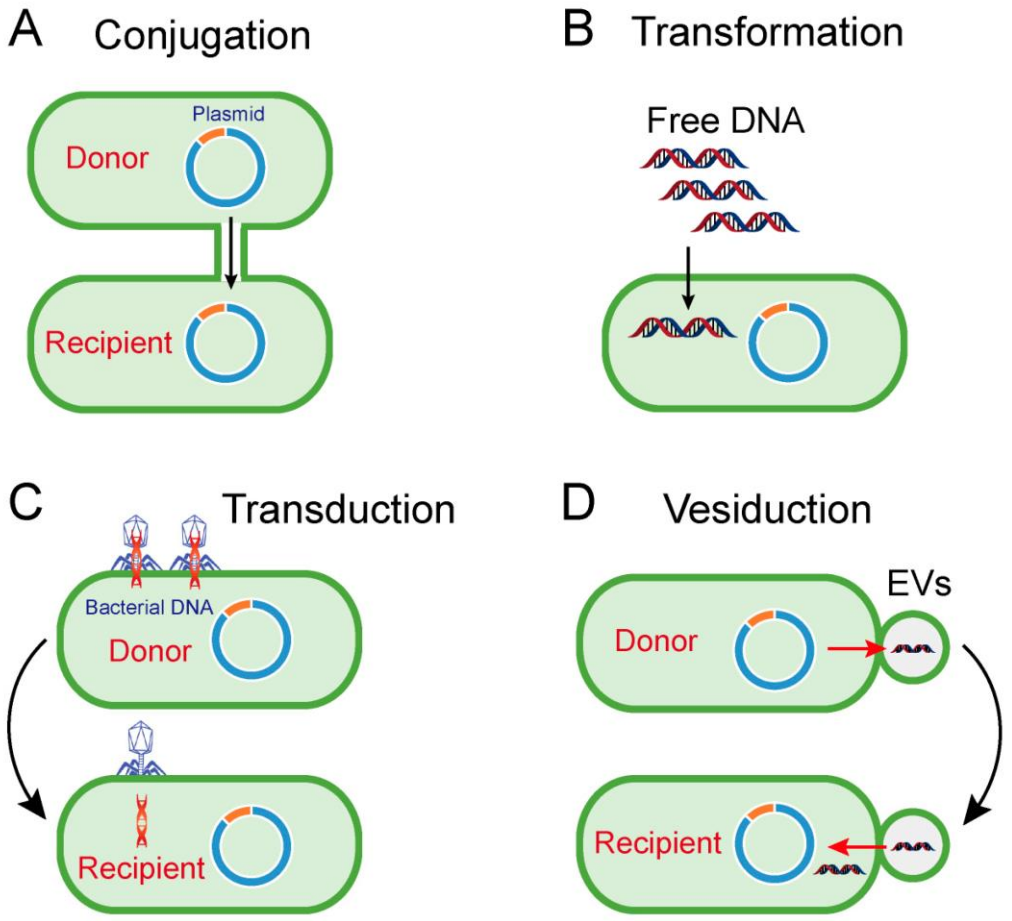


Established Synthetic Urine Growth Curve with *Rhodococcus opacus PD 630*



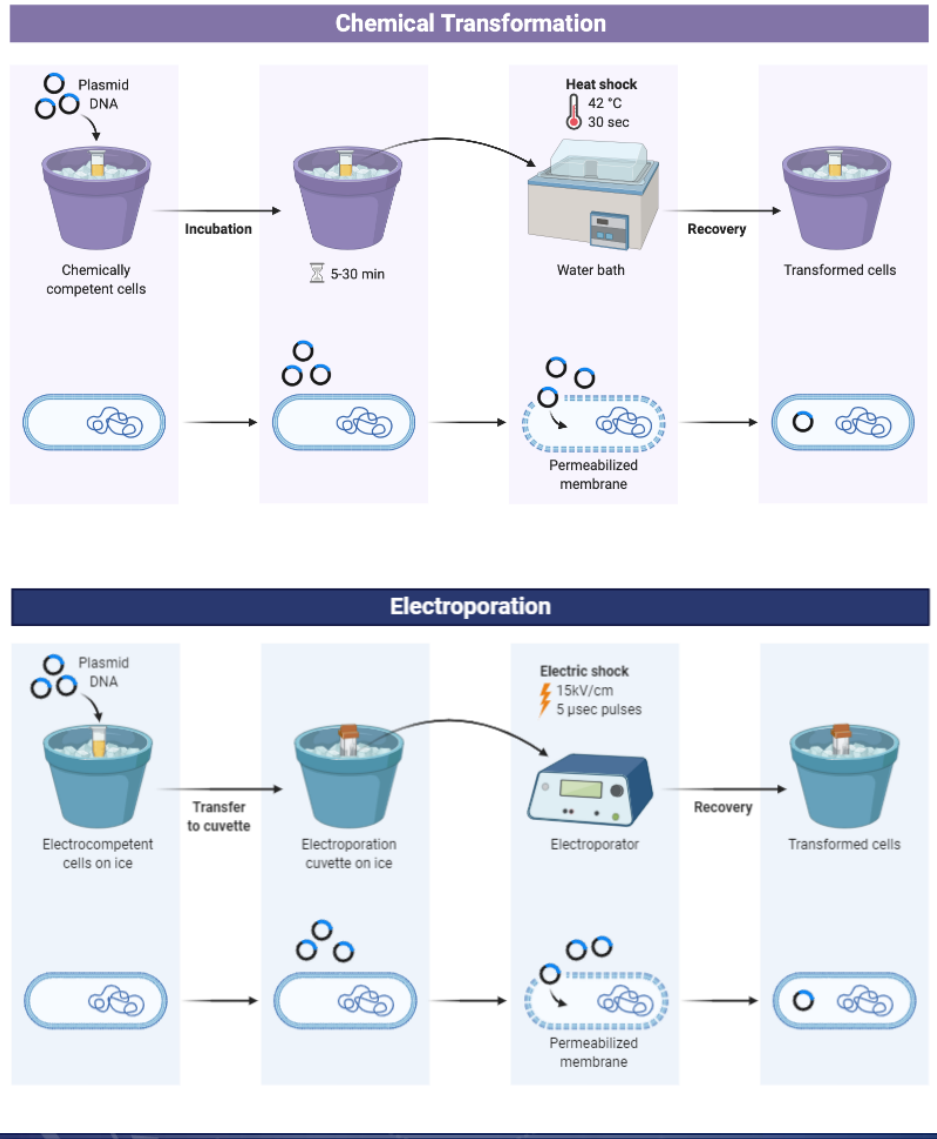
Introduction of DNA into Cells: Modes of Transformation and Electroporation Optimization

Introducing a DNA gift



https://www.mdpi.com/2076-2607/8/8/1211/htm

Chemical Transformation vs Electroporation



https://app.biorender.com/biorender-templates/figures/5c95180bc2753f33003dacd3/t-5fda6e1f8c41295288bbf23b-bacterial-electroporation-transformation https://app.biorender.com/biorender-templates/t-5fda6dba8c41295288bbf0b7-bacterialchemical-transformation

Optimizing Electroporation for *R. opacus* Competent Cells

Basics of creating competent cells

- Start of seed: 2 mL, 30 C, 150 rpm, 1 loop of bacteria
  - Increase to 58 mL solution
- Optical density (OD) 0.5, 30 C, 150 rpm
- Washed twice: 10 minutes intervals, air 4 C, samples 4 C, 2200g, ice cold water. Concentrated 20-fold.
- Mixed with 10% glycerol at respective fluid amounts for NB or LB procedure. (800 µL for NB and 400 µL for LB)

Basic electroporation requirements

- Amps: 25 µF
- Volts: 2000 V
- Resistance: 600 Ω
- Concentration of experimental solution: 0.1 µg/ mL pBAV1K-gfp
- Pass condition: at least 3 viable colonies of bacteria present

NB base procedure

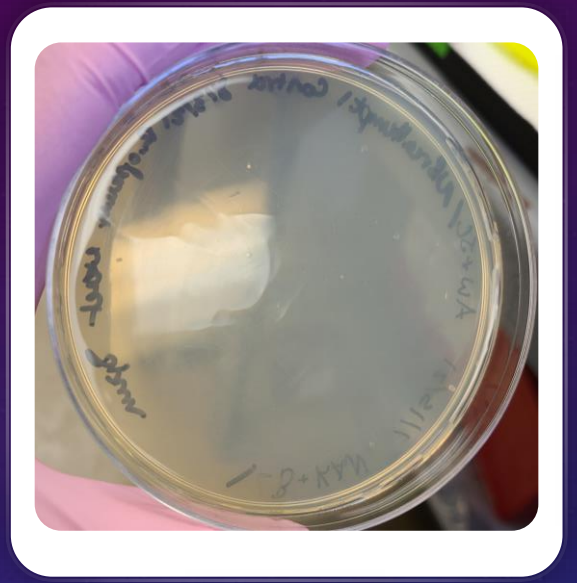
- Time in heat and temp: 40 C, 5 min
- Ice or no ice: no ice
- Predicted time constants from literature: 3-5 ms
- Regeneration medium and time: 600 µL NB, 30 C, 4 hrs
- Predicted time to see results: 3-4 days

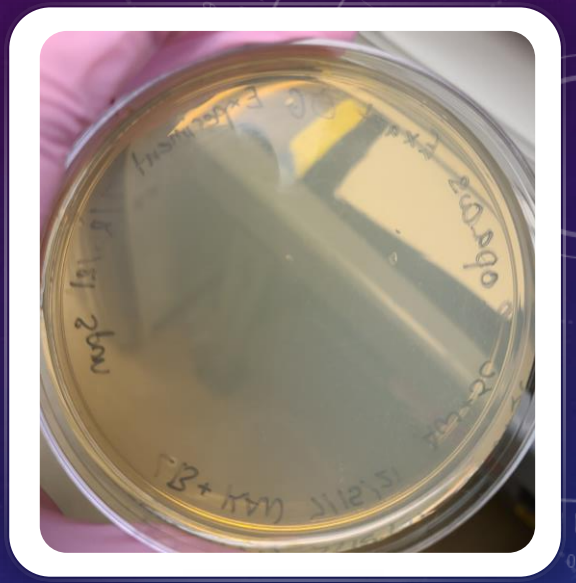
LB base procedure

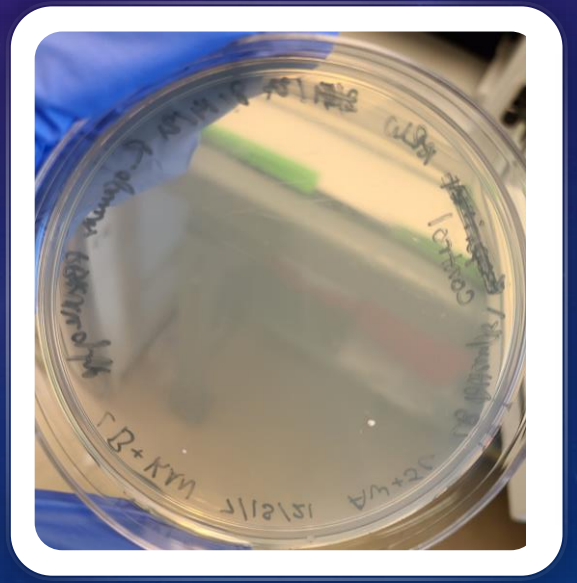
- Time in heat and temp: 40 C for 5 minutes
- Ice or no ice: ice, 10 minutes
- Predicted time constants from literature: 6ms
- Regeneration medium and time: 800 µL SOC for 4 hr
- Predicted time to see results: 3-4 days

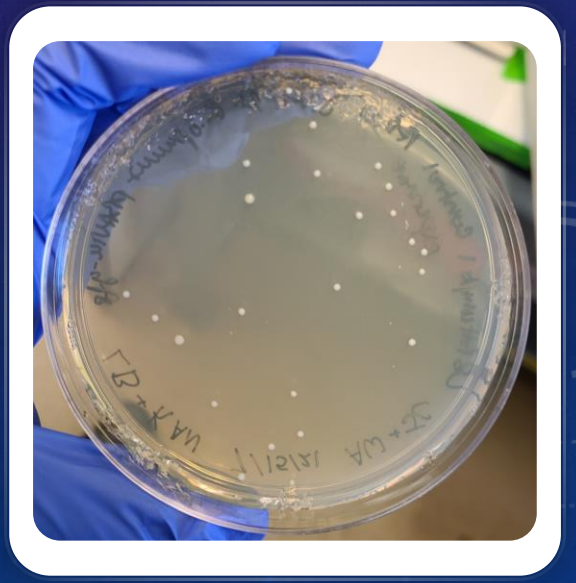
Electroporation Results

- Results from the Nutrient Broth (NB) Experimentation
  - Average colonies per plate: 0-2
  - Time for growth: 9-11
  - Time constant range: from 8 to 12.7 ms for the experimental bacterial samples and 10 -12.7 ms for the control samples
- Results from the Luria-Bertani Broth (LB) Experimentation
  - Average colonies per plate: 3-7
  - Time for growth: 5.25
  - Time constant range: 12.2 ±0.13 ms for control and 12.4 ±0.53 ms for experiment
- Luria Bertani Broth recommended for further experimentation to establish pBAV1K-efe







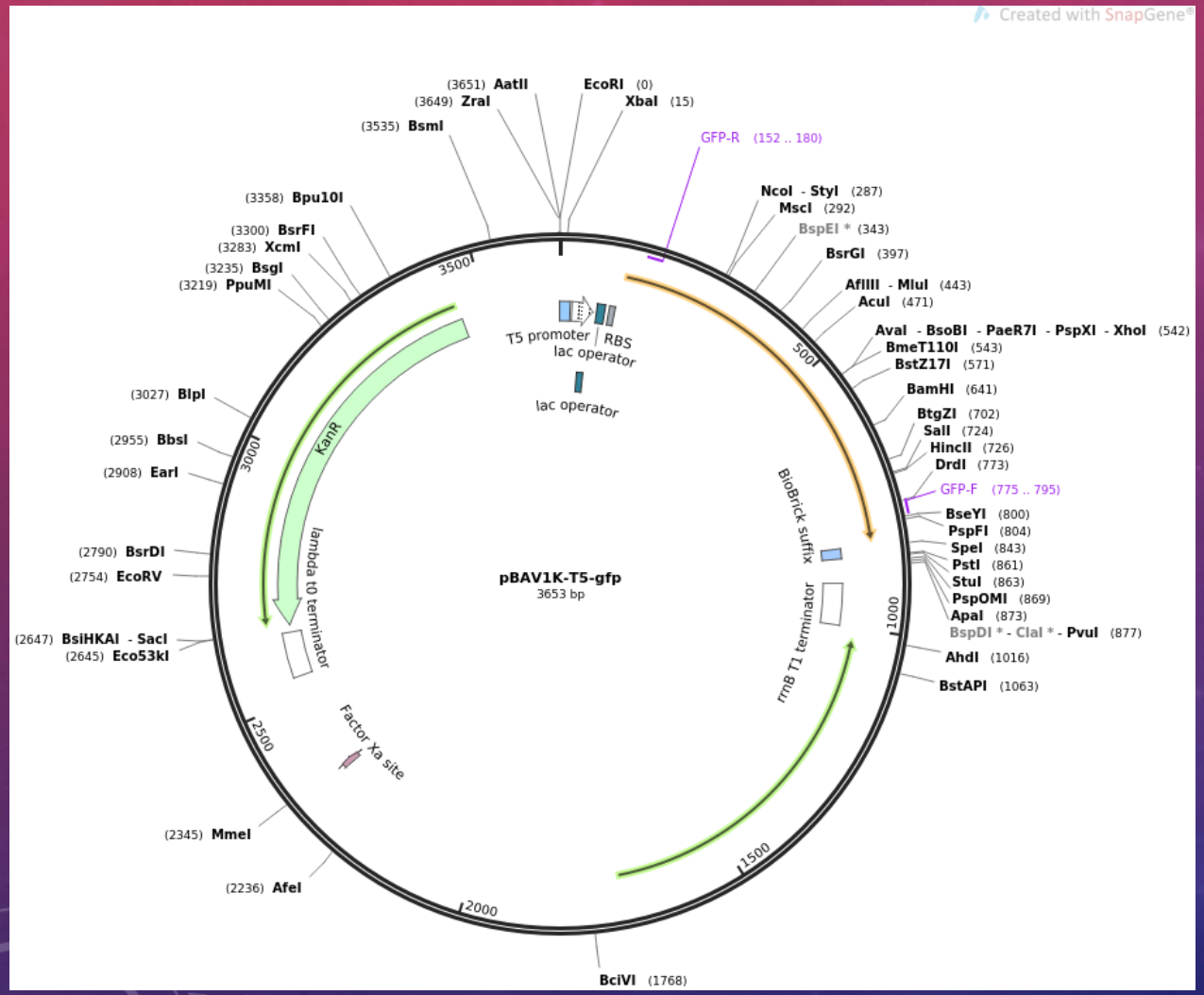


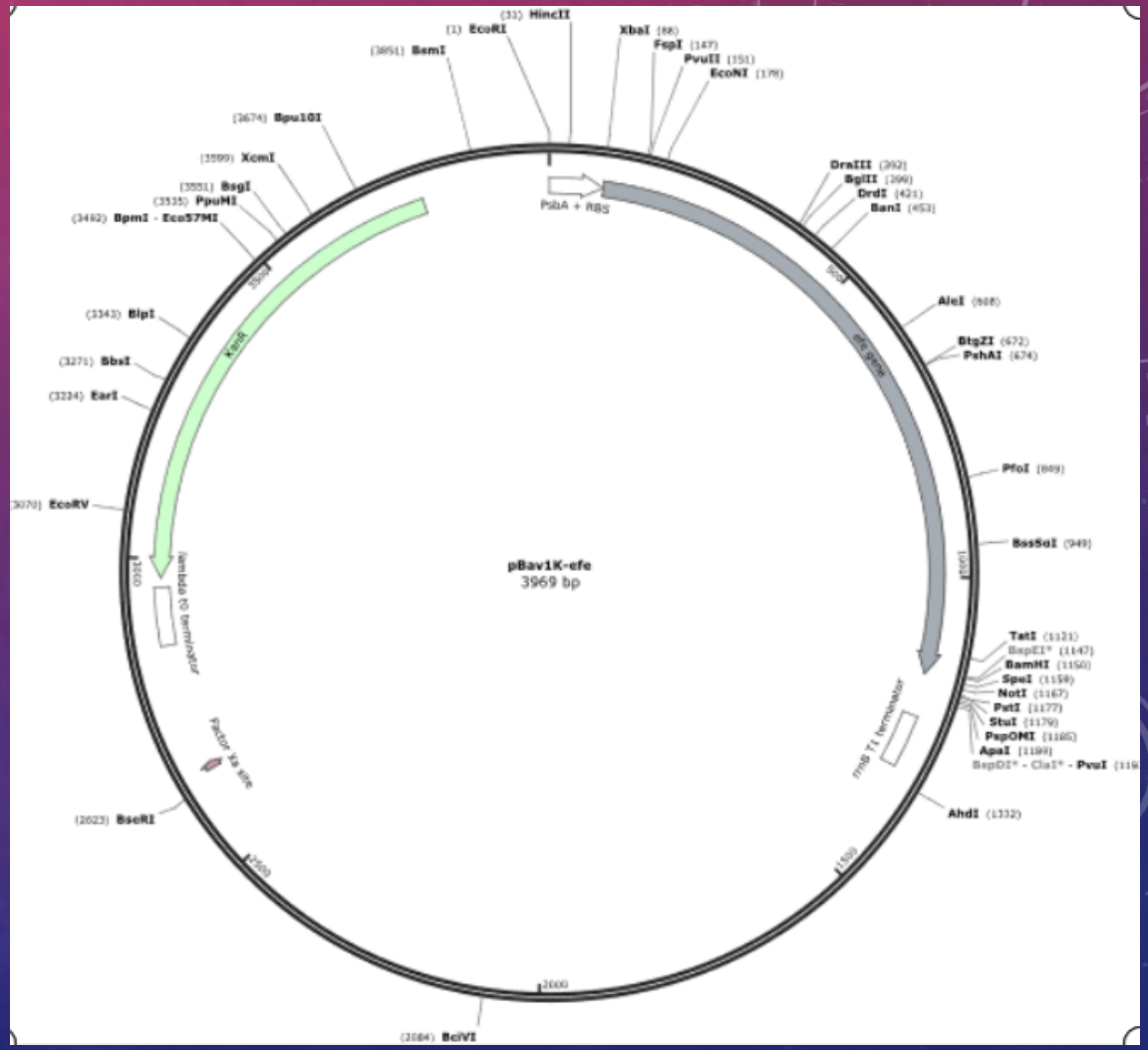
Producing the Cloning Plasmid and Progress Made

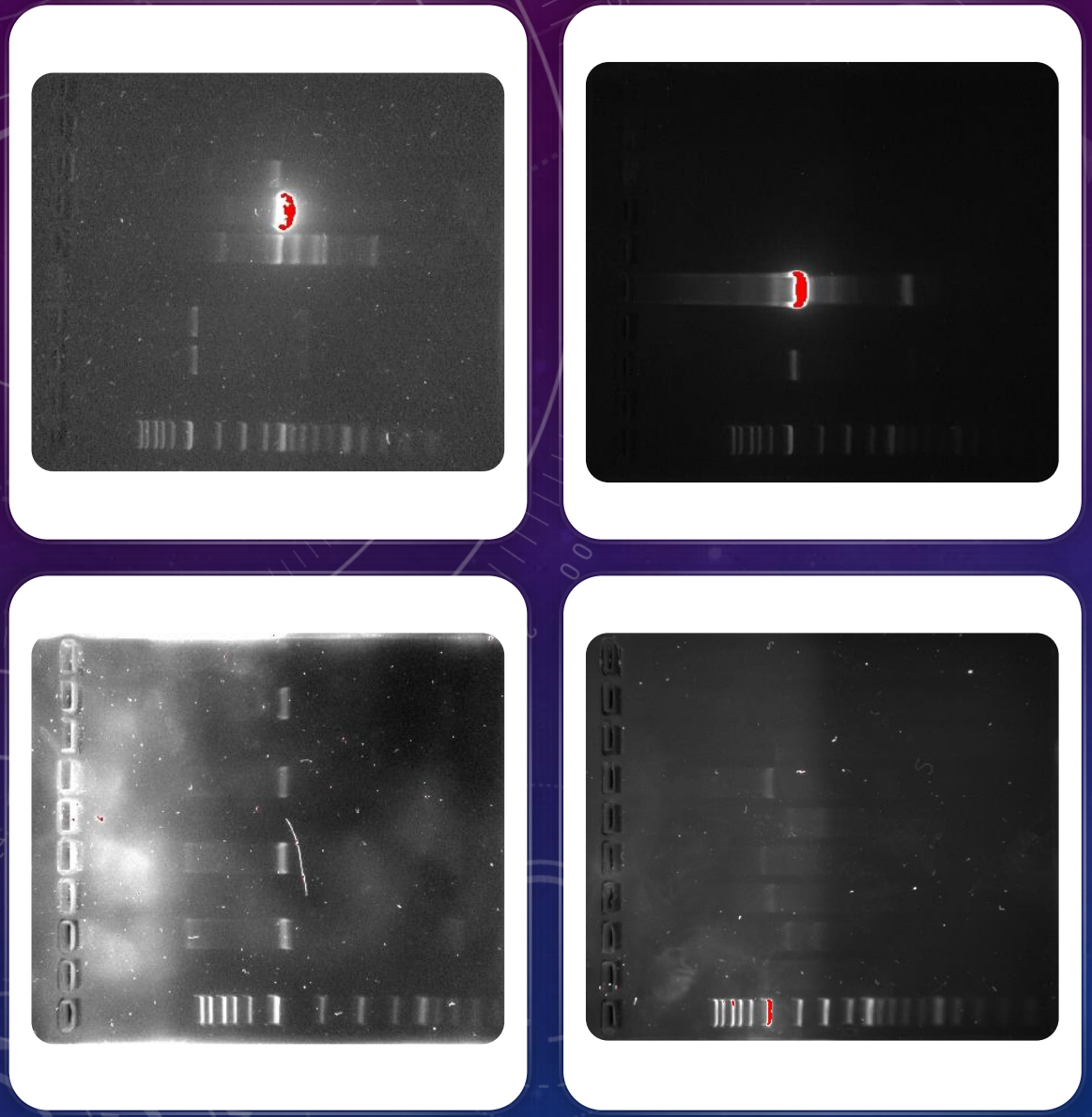
Making the Cloning Plasmid

- The Backbone
  - Developed from digesting pBAV1K-T5-gfp with EcoRI and SpeI and extracting it from an agarose gel. Provides structure to the plasmid as well as Kanamycin resistance for selectivity
- Efe gene
  - Previously isolated from a former colleague. Theoretically allows the *R. opacus* PD630 to use a TCA cycle intermediate produced from its fatty acid degradation, α-Ketoglutarate, into ethylene
- PsbA promoter and RBS
  - Both complementary strands purchased and applied to heat to combine into a double-stranded insert. Being a high-fidelity promoter and ribosome binding site, this would provide a higher chance for the plasmid to be processed in transcription and translation.
- Cloning procedure:
  - Follows NEBuilder® HiFi DNA Assembly Cloning Kit's procedure for 4-6 Fragment assembly with a higher ppm ratio than recommended.

pBAV1K-efe VS pBAV1K-T5-gfp Plasmid: Schematics and Identification Measures







Results of Plasmid Cloning Procedure

Moving Forward: Transformed Cell Growth Studies, Ethylene Detection, and Product Optimization

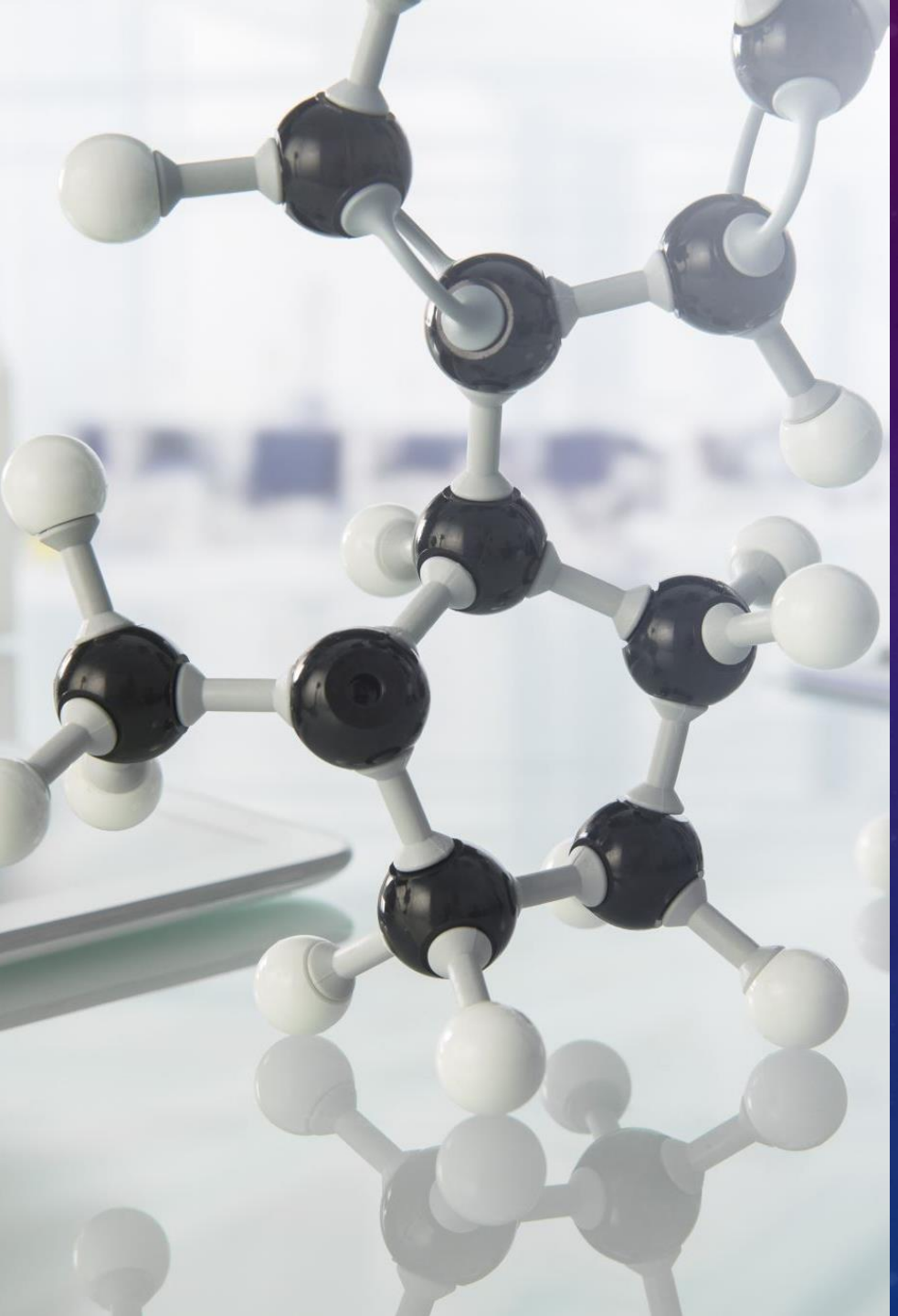
Growth Studies Post Plasmid Introduction

Procedure:

- Like previous growth media experiments
  - Using the same conditions as the wild-type growth curves, record the OD of the samples every 6 hours to determine the growth throughout the media.
  - Differences in the growth media content may occur, as the media will now contain Kanamycin in a 50 µg/mL concentration.

Anticipated outcome:

- The overall OD is predicted to reach a smaller maximum OD based on the results observed from the regeneration time for the electroporated test cells. Fatty acid accumulation and (potentially) ethylene production could show an increase within the synthetic urine when compared to the nutrient rich LB media.



Gas Chromatography and Ethylene Detection

- Calibration of the Gas Chromatographer (GC) and the GC's Model:
  - Calibration gas: Ethylene
  - Gas Chromatographer: Agilent Technologies 7890B GC System
- Ethylene Detection and Standard for Positive Result:
  - Would run the sample in the GC and observe the graph created for peaks at the location of ethylene's peaks in literature and compare the literature to our known graph from running the sample.
  - Positive result would be any peak at ethylene's calibration locations.

Concluding Statements

Future Work

Procedure modification and implementation of the cloning plasmid, pBAV1K-efe, into competent *R. opacus* PD 630 cells

Growth curve experimentation to establish differences in bacterial development post plasmid introduction

Ethylene detection through gas chromatography

How could the Project have been improved? What other avenues could we try if this strain cannot produce our results?

- Area of improvement:
  - Preciseness of backbone excision
  - Repeat testing to prevent any results due to contaminants.
  - Recharacterize the plasmid and define expected restriction enzyme cut lengths for plasmid identification.
  - Research more into the transformation process into Rhodococcus opacus as it was not clearly written in literature and could be a source of error for the cloning plasmid's electrophoresis results.
  - Exact composition of gel loading buffer for samples
  - Concentrating DNA for less waste of material.
- Potential avenues to explore:
  - Different backbone
  - Different strain
  - Alternative cloning method
  - Different conditions for ethylene detection and media growth

Acknowledgements

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Resources

- Agilent Technologies. *Agilent 7890B Gas Chromatograph Operation Manual.* https://www.agilent.com/cs/library/usermanuals/Public/7890B_Operation.pdf.
- BiteSizeBio. (2016, July 16). *E. coli Electroporation vs Chemical Transformation*. https://bitesizebio.com/10297/ecoli-electroporation-vs-chemical-transformation/.
- 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.
- GenScript. *What is DNA Transformation*. https://www.genscript.com/what-is-dna-transformation.html.
- 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.
- 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.

THANK YOU!

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