ETHYLENE PRODUCTION FROM *E. COLI*

A Senior Scholars Thesis

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MATTHEW GERICH

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

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Approved by:

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ABSTRACT

Ethylene Production from *E. coli*. (May 2012)

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Ethylene is one of the most important and commonly used chemicals. Production of ethylene from a renewable resource would greatly reduce the amount of petroleum used in chemical processing. This would be a great help to reducing green house gases.

There are several ways to produce ethylene from *E. coli*. One could modify the genome of the bacterium to naturally produce ethylene as a part of its growth cycle. Another method is to make the bacterium produce an enzyme that catalyzes a reaction to produce the desired product. This enzyme would generate ethylene from a compound the bacterium naturally produces. The use of the Ethylene Forming Enzyme from *Pseudomonas syringae glycinea* greatly simplifies the ethylene forming process.

The Ethylene Forming Enzyme was first isolated from *Pseudomonas syringae glycinea* (MAFF 302676 and MAFF 301683). It was then ligated into a plasmid and

electrophorated into *E. coli*. Once the plasmid was in *E.coli*, the bacterium naturally produced and secreted ethylene gas as it grew.

Once the bacterium was able to produce ethylene, it was further modified to grow on corn stover. This allowed for ethylene production from a bio-waste feedstock. After corn stover growth was achieved the *E. coli* was further modified in an attempt to increase ethylene yield.

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The research presented here would not have been possible without the help of Dr. Katy Kao and graduate student James Winkler. Both provided valuable insight into the process used and helped with troubleshooting any problems that occurred. The use of Dr. Katy Kao's research lab was also a necessity where my research could be performed and with minimal financial cost to the lab.

NOMENCLATURE

°C	Degrees Celsius
DNA	Deoxyribose Nucleic Acid
DNTP	Deoxyribose Nucleotide
g	Grams
h	Hours
MAFF	Ministry of Agriculture, Fisheries and Food
mg	Milligrams
mL	Milliliters
mV	Millivolts
nL	Nanoliters
PCR	Polymerized Chain Reaction
s	Seconds
TAE	Tris Base, Acetic Acid, Ethylenediaminetetraacetic acid Buffer
TCA	Tricarboxylic Acid
μL	Microliter

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CHAPTER I

INTRODUCTION

Chemical processing today relies heavily on crude oil as a primary feedstock. The increase in technological advancement and population has continued to strain our current and future energy supply. The large use of fossil fuels is starting to have an effect on the environment. By limiting the amount of petroleum used in chemical manufacturing we can help to lower our reliance on fossil fuels. Ethylene is one of the largest chemicals produced in industry. It is also produced from petroleum. By developing an alternative production process that does not use petroleum we can both lessen our reliance on fossil fuels and our production of green house gases.

Through bioprocess engineering bacteria has been modified to produce various products. These new production pathways have helped to provide alternatives to current methods. One primary example is fuel in the form of ethanol, butanol, and iso-butanol. These fuels are commonly produced from petroleum. By modifying bacteria to produce these fuels we can now produce them without having to use fossil fuels. Similar methods that are used to modify these bacteria can be used to modify bacteria to produce any chemical we desire.

This thesis follows the style of Metabolic Engineering.

Bacteria are commonly modified in two main ways. One way involves the altering of the bacteria's metabolic pathway so that when it grows it now produces new chemicals that we desire. The other way is to insert a plasmid that contains a coding for a new enzyme or gene that will allow the bacteria to produce the target chemical. The second of these methods is easier to accomplish and normally requires less steps than the first method.

Researchers in China and Japan have already successfully modified *E.coli* to produce ethylene using what is called the Ethylene Forming Enzyme. This enzyme is found in a naturally occurring plasmid in some strains of the plant pathogen *Pseudomonas syringae*. By isolating the gene found in *Pseudomonas syringae* and inserting it into a plasmid, researchers have been able to successfully modify strains of *E. coli* and other bacteria to produce ethylene (Chen et al., 2010). The Ethylene Forming Enzyme is produced in the cell and allows for the transformation of 2–oxoglutarate to be transformed into ethylene (Weingart et al., 1999). The ethylene gas is then secreted from the cell and can be captured from the gas above the growing bacteria.

Current yields of ethylene using the Ethylene Forming Enzyme plasmid is very low at 10 μL ethylene/(mg cells·h) (Ishihara et al., 1995). Various methods to improve the process yield have been tried by several groups. This paper will verify that production of ethylene is in fact possible using bacteria growing on a bio-waste feedstock. It will then present several ways to try and improve ethylene yield to make the process practical.

This involves removing inhibitors, over expressing process reagents, and modifying the gene insert.

CHAPTER II

METHODS

Materials

This section describes how all bacteria strains and any relevant chemical reagents were obtained.

Bacteria

Four strains of *Pseudomonas syringae* were obtained for further study. Two strains PG-11 of *Pseudomonas syringae pv. glycinea* and HB10Y of *Pseudomonas syringae pv. phaseolicola* were obtained from Dr. Gross at Texas A&M University. After further study two more strains of *Pseudomonas syringae pv. glycinea* MAFF 301683 and MAFF 302676 were obtained from another research group. The plasmid was obtained from a PYG2 bacteria strain in lab.

Chemical Reagents

The LB media is created the laboratory stock of Tryptone, Bacto Agar, Sodium Chloride, Yeast Extract, milliQ water, and Ampicillin. Gels for electrophoresis were created with Agarose, 0.5X TAE and Ethidium Bromide. Reagents for electrophoresis include 2-log ladder and 6x loading dye. For the PCR molecular grade water, ONE TAQ polymerase, ONE TAQ buffer, and 10mmolar DNTPs were used.

Equipment

Various sized pipettes, media bottles, and flasks are used in this experiment. Also sterile petri dishes were used four making bacteria plates. PCR tubes are also used for use in the PCR machine. For electrophoresis gels a plastic gel plate is used.

Instrumentation

This section describes all instrumentation and computer programs used for the formatting of ethylene from E. coli.

Machines

Several in laboratory machines were used during this project. Several clean hoods are used to create a sterile environment for bacteria manipulations. A PCR (Polymerase Chain Reaction) Machine is used to amplify DNA fragments. An electrophoresis machine is used for gel electrophoresis of DNA. A Gel-Doc XR is used to record images of gels. Freezers are also used to store bacteria and DNA templates. An autoclave is used to sterilize containers and media. A Bunsen burner is used to create a sterile environment for pouring plates. Several incubators and shakers are used throughout the experiment for bacteria growth.

Software

The software used is the Gel-Doc XR software that is required to use the Gel-Doc XR machine. Also the software for the autoclave is necessary to select the autoclave cycle.

Methodology

This section describes in detail the methods used to obtain the results presented in this report.

Preparation

Before research could begin several materials must be prepared for use. LB media is prepared by combining 5g NaCl, 2.5g Tryptone, and 2g Yeast Extract to 500mL of miliQ water. If making plates 7.5g of Bacto Agar is also added to the mixture. This is then autoclaved by selecting the liquid 20 program on the main screen. After the autoclave is complete the media is ready for use. If making plates with an antibiotic the media must be placed in a water bath set to 35°C and left for 30 minutes before adding the appropriate amount of antibiotics. In our case 1mL of Ampicillin (100µg/mL) is added to make the LB-Amp plates.

After the addition of the appropriate antibiotics if any, the media can then be poured onto plates. This is done using a Bunsen burner to create a sterile desk as you pour the plates around the base of the burner. The plates are then covered and left out overnight after pouring. They are placed in the fridge the next morning for later use.

In order to make gels for electrophoresis 0.5g Agarose is added to 50µL of 0.5X TAE. The solution is then microwaved for 1 minute and 10 seconds and swirled to make sure all Agarose has dissolved. Then 4µL of Ethidium Bromide is added and mixed. Gels are poured using a gel mold with the plate at the bottom.

DNA Replication

In order to replicate the DNA of the EFE Gene a PCR reaction must be performed. This is performed by adding 36.75µL of molecular grade water to a PCR tube. Using a 10µL pipette bacteria is obtained off a plate and pipetted into the water. The solution is then boiled using the BOIL PCR program at 98°C for 10 minutes. After boiling 0.25µL of ONE TAQ Polymerase, 10µL of ONE TAQ Buffer, 2µL of primer template, and 1µL of DNTPs is added to the solution.

Then the solution is placed back in the PCR machine and the ONETAQ PCR program is run. This program has 7 main steps:

1. Heat to 94°C and hold for 30 seconds
2. Heat to 94°C and hold for 30 seconds
3. Gradient temperature 50°C and 50.7°C and hold for 30 seconds
4. Heat to 68°C and hold for 30 seconds
5. Repeat steps 2-4 for 30 cycles
6. Heat to 68°C and hold for 5 minutes
7. Hold at 10°C forever

After the PCR program has finished the copied DNA is removed from the machine. In preparation for electrophoresis 2µL of 2-log ladder is added to one of the wells in the gel. Before addition of the copied DNA to the gel it must be mixed with the 6x loading

dye. This can be performed by adding a 1µL drop of loading dye onto parafilm. Then 5µL of the DNA is pipetted up and down onto the loading dye until mixed. After being mixed it is added to the well in the dye. The electrophoresis is performed at 135 mV for 16 minutes.

Once the electrophoresis is finished the gel is removed and placed on the Gel-Doc XR screen. The gel image can be viewed by opening the Gel-Doc XR software and clicking the button for UV light and auto expose. After the image appears it can be saved. This is used to verify the PCR reaction was successful.

CHAPTER III

RESULTS

The pathway for production of ethylene via the ethylene forming enzyme is depicted in Figure 1.

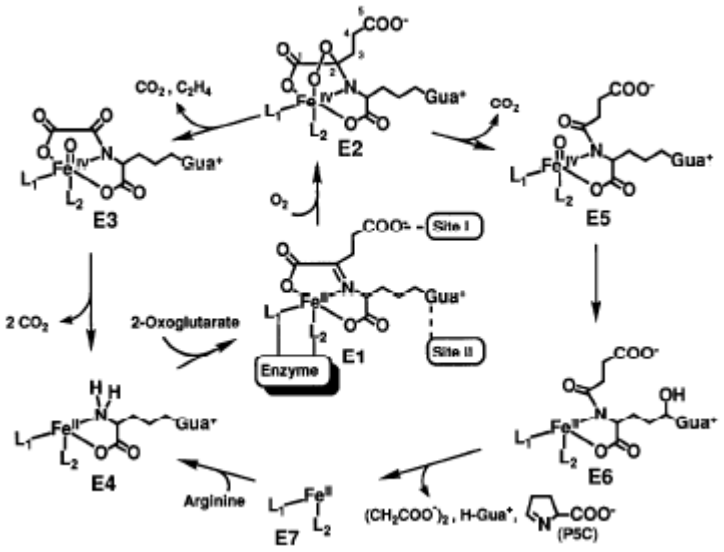

Figure 1. Ethylene formation via EFE and 2-Oxoglutarate (Nagahama et al., 1998).

This pathway allows for Ethylene to be produced directly from *E. coli* because 2-Oxogultarate is a product in the tricarboxylic acid cycle (TCA cycle). Because ethylene is a gas at room temperature it will be excreted by the cell and bubble out of the media for capture.

Many different PCR reactions were performed to attempt to amplify the gene that codes for the EFE from various strains of *Pseudomonas syringae*. Table 1 shows the strains of *P. syringae* tested and those that were proved to contain the EFE gene.

Table 1. Results from experimental and control runs.

Strain	EFE gene
PG-11 *Pseudomonas syringae pv. glycinea*	No
HB10Y *Pseudomonas syringae pv. phaseolicola*	No
Pseudomonas syringae pv. glycinea MAFF 301683	No
Pseudomonas syringae pv. glycinea MAFF 302676	Yes

Only one of the four strains was determined to have the EFE gene. This gene was copied via a PCR reaction.

Several PCR polymerases and reaction conditions were also performed. Table 2 lists these reactions and the amplification obtained from *Pseudomonas syringae pv. glycinea* MAFF 302676.

Table 2. PCR reaction conditions.

Polymerase	Melting Temperature (°C)	Melting Temperature Duration (s)	Amplification
TAQ	50-75	30	None

Table 2. Continued.

Polymerase	Melting Temperature (°C)	Melting Temperature Duration (s)	Amplification
TAQ	55-75	120	None
TAQ	50-55	120	Small
Phusion	50-75	30	None
Phusion	50-75	120	None
ONETAQ	50-75	30	None
ONETAQ	51.4-75	120	None
ONETAQ	50-50.7	120	Large

The amplification of the EFE gene was then narrowed down to abnormally cold melting temperatures for the PCR reaction. The gene was then obtained from only *Pseudomonas syringae pv. glycinea* MAFF 302676 through PCR with ONETAQ PCR polymerase with a melting temperature of 50 °C.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

The ethylene forming enzyme provides a very convenient and useful pathway for producing ethylene from *E. coli*. The enzyme itself can be found within several species of *Psuedomonas syringae*. *Psuedomonas syringae* naturally produces the ethylene forming enzyme from a DNA marker on an internal plasmid (Sato et al, 1997). In this experiment the *Pseudomonas syringae pv. glycinea* MAFF 302676 strain was determined to contain the ethylene forming enzyme gene. The other bacteria tested, PG-11 *Pseudomonas syringae pv. glycinea*, HB10Y *Pseudomonas syringae pv. phaseolicola*, and *Pseudomonas syringae pv. glycinea* MAFF 301683 did not contain the gene.

Amplification of the ethylene forming enzyme gene was performed by colony PCR. The DNA polymerase used to amplify the gene was ONETAQ DNA polymerase. Both TAQ and Phusion DNA polymerases failed to amplify the gene. In particular the PCR reaction was run at abnormally low temperatures (50 °C) and long time lengths (2 min extension time).

Conclusions

This research confirmed the existence of the ethylene forming enzyme gene. It also verified that the gene can be copied and inserted into another bacterium. The experiment established protocols for future work with the ethylene forming enzyme gene.

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