The Reduction of Carbon Dioxide to Methanol through *Escherichia coli* Phase 1: Engineering an Obligate Aerobe

by: Caitlin O'Brien

Submitted to:
Frank LaBanca, Ed.D.
Science Department Chair
Oxford High School
June 10, 2010

Abstract

Global warming has brought about a push toward alternate energy in recent years. Converting carbon dioxide to methanol through *E. coli* would provide a renewable carbon neutral energy source. The pathway necessary to facilitate this conversion is one of two present in *E. coli*, respiratory and fermentative. *E. coli* were irradiated with short wave ultraviolet light to induce mutation, leaving only the desired pathway intact. In order to screen for fermentative pathway mutation, a novel phenol red indicator method was developed using a 96- well plate format. Mutants unable to survive without oxygen were selected using parallel plating.

Introduction

Global Warming

Global warming is one of the major issues facing humankind today. There are many theories surrounding the cause of this widespread problem, but many agree that greenhouse gases are one of, if not the only, root of global warming. Greenhouse gases do exactly as the name implies, blanket the planet and prevent the sun's heat from leaving as it should. This blanket leads to countless environmental issues, including (but not limited to) melting ice caps, and unseasonable temperatures (Miller, 2010). In the past few years there has been a great push toward cleaner burning fuel and conservation for lower greenhouse gas emissions, but the planet continues to be stifled by humankind's carelessness. Harmful gases continue to pour out of engines and factories, polluting the atmosphere and, if continued unchecked, this trend will lead to the utter ruin of not only man but all of the other creatures that share the earth.

Carbon Dioxide

Carbon dioxide is one of the harmful gases that are pumped into the air every day in unnatural amounts. It can be found wherever humans have touched, not only as a result of respiration but as a result of the continual drive for bigger and better machines and production lines, namely cars and factories. While plants use carbon dioxide for photosynthesis, there is only so much that can be used. Deforestation and other destructive human activities are continuously shrinking the number of plants alive to use the constantly increasing amount of carbon dioxide (Postlethwait, 2006). While the number of plants is continuously shrinking, the number of humans is growing every year, creating more carbon dioxide with every breath (Postlethwait, 2006). This leaves the

planet in a very one sided competition, and frankly, carbon dioxide is winning hands down. Since all of the carbon dioxide produced cannot be utilized by the plants and other organisms that survive by way of photosynthesis it collects in the atmosphere, baking the planet below through the greenhouse effect. The greenhouse effect is the heating of the Earth due to an accumulation of gases in the atmosphere which trap the sun's heat and lead to global warming.

Renewable Energy

Renewable energy sources are necessary for the future of humankind. Every year it becomes more apparent that the world's supply of oil is not inexhaustible. Even so, humankind's demand for this commodity is in no way decreasing. Current alternative fuels have proven to be either too costly or impractical (University of Oregon, 1999). Wind turbines can cost billions to build, but once built these giant towers can only produce electricity for a limited area. This would be fine except for the fact that the type of wind that is necessary to make the cost of building the turbine worthwhile is not present in many places (University of Oregon, 1999). Even in places where the wind turbine idea is viable people will sacrifice the prospect of clean energy due to the fact that many people believe that wind turbines are lacking aesthetically. Dams are no better and actually carry their own set of environmental concerns, including the destruction of surrounding habitats. These energy sources also cost an incredible amount of money to build, and can only be built where there are rivers that run deep enough and strong enough for the dam to be worthwhile (Ryan, 2009). Needless to say, river that meet the necessary parameters do not run in enough places to provide energy to the entire world. Solar energy is also an impractical energy source, unless new developments are made in the field. As of today, solar energy is too expensive to be

feasible, not to mention the fact that the panels need to be exposed to the sun in order to produce energy (University of Oregon, 1999). Solar energy therefore can only be used in places that do not get frequent precipitation, which would block the sun's rays and make the costly panels useless.

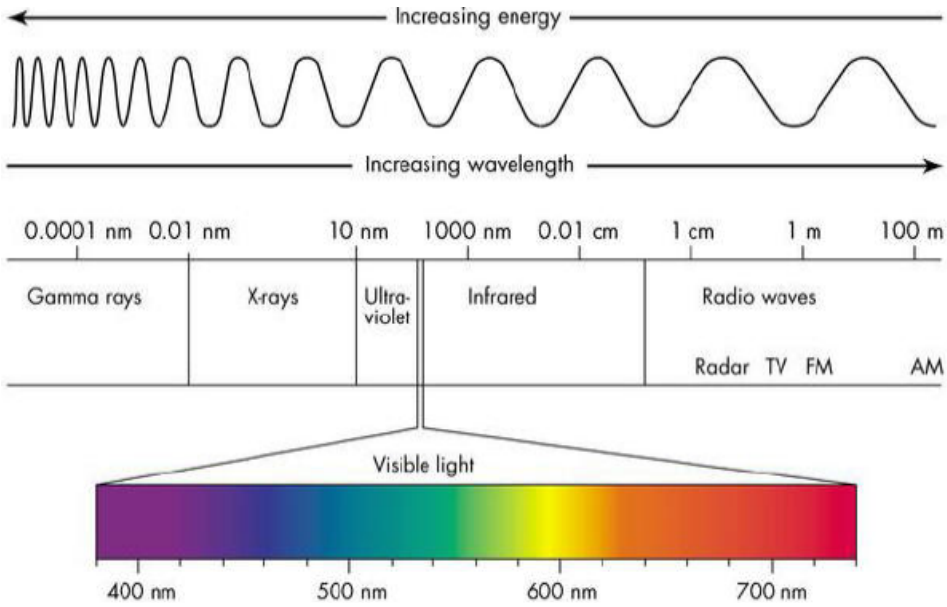
Methanol

Methanol has proved to be a viable alternative to gasoline in engines. It is a cleaner burning fuel than gasoline and, if produced without the use of fossil fuels, could offer the answer to the need for renewable energy (Woo, 2001). Though the hydrogen fuel cell has been a great object of conversation among those developing new fuel sources in recent years, there are quite a few risks inherent in the use of a highly combustible gas. Many people would not be comfortable with the prospect of using such a volatile source of energy since just about everyone has heard about the Hindenburg incident (Krystek, 2001). Methanol, on the other hand, is actually less flammable than gasoline making it safer to use. Unfortunately, methanol also does not contain as much energy as gasoline. "Because methanol has a lower energy content than gasoline, it takes 1.65 gallons of M85 to provide the same amount of energy or range as in one gallon of gasoline. However, methanol's octane rating is higher, which gives better performance and acceleration" (Methanol Institute 2009). These pros outweigh the cons, which is why methanol is the fuel of choice for many vehicles, such as racecars and go-carts.

Ultraviolet Light

Ultraviolet light has long been known for its mutagenic properties. It is the type of light that has energy just above that of visible light (see Figure 1). This light is known for its mutagenic

properties, especially those resulting from prolonged exposure to the sun (sunburn). The very property that causes so many pains in the summer also proves to be greatly helpful in the sterilization of both tools and foodstuffs. Many microorganisms are killed by short wave ultraviolet light, but in lower doses this light can mutate these organisms, including *E. coli* (Jamison, 1987). Ultraviolet light works to mutate bacteria by damaging the DNA of the organism exposed. Many scientists have used this property of ultraviolet light, coupled with the known genome of *E. coli* to better learn about mutations. When subjected to ultraviolet irradiation, *E. coli* can be mutated in many different ways according to the scientist's wishes. Though the actual mutations are not easily predicted, a scientist can select which mutant he wants after irradiating the bacteria. The mutants can be selected by placing the bacteria in various growth environments and selecting for the desired characteristics.



http://sites.google.com/site/blozzonchemistry/home/honors-chemistry-period-c/atomic-theory-and-electron-structure

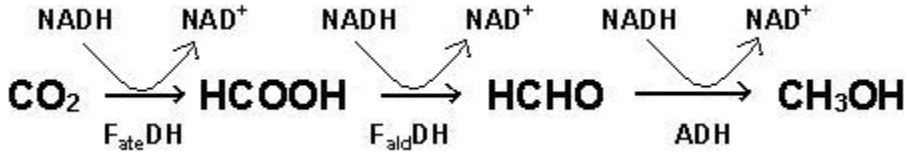
Figure 1: The Light Spectrum

Making Methanol Using Enzymes

In 2001, the scientists Robyn Obert and Bakul C. Dave demonstrated that methanol could be created through the use of three enzymes; formaldehyde dehydrogenase, formate dehydrogenase, and alcohol dehydrogenase. The scientists incorporated these three enzymes into a sol-gel matrix along with NADH. When electricity was run through the gel, methanol was produced. This experiment was a breakthrough on the front of using methanol as an alternative fuel source since no fossil fuels were used in the production of the methanol. Unfortunately, this method is impractical and time consuming. The cost of the materials necessary to produce methanol is far too high to allow this gel to be the methanol producer of the future. Conventional methanol production is much cheaper and more efficient; the only negative regarding this method is the fact that the majority of methanol produced through conventional means is made with the use of fossil fuels. Gel made methanol cannot compete with this production method, since “it costs $114.90 set up one reaction and it would take over 22 years to get one mole of methanol, in contrast to conventional production, which only costs $29.00 per gallon” (Woo, 2009). Currently, the gel method is far too expensive to be considered for any purpose other than satisfying scientific curiosity. Even the seeming greenness of the gel method is somewhat too good to be true. Though the gel method does not use fossil fuels directly, unless the electricity used comes from a green source, this method of producing methanol still relies on the continuing use of fossil fuels.

Enzymes Used

Alcohol dehydrogenase reduces methanol to formaldehyde in the methane oxidation pathway. Formaldehyde dehydrogenase works to reduce this formaldehyde into formate, which is then reduced in turn by formate dehydrogenase. This enzyme reduces the formate to carbon dioxide. It is through the reversal of this pathway that these enzymes can be used to make methanol from carbon dioxide.



http://www.biochem.arizona.edu/classes/bioc462/462bh2008/462bhonorsprojects/462bhonors2000/jwoo/index/html

Figure 2: Metabolic Pathway Transforming Carbon Dioxide to Methanol

Genetically Modified Organisms

Many genetically engineered and modified organisms have been used in recent years to aid humankind. Genetically modified foodstuffs are some of the most controversial of these organisms, but they also have the potential to be some of the most beneficial. Rice, for instance, is a staple crop in many places across the face of the globe. Unfortunately, conventional rice does not contain all of the vitamins necessary for a healthy diet most noticeably beta-carotene. A lack of this vitamin in the diets of people who rely on rice for daily sustenance can lead to blindness and various other medical concerns. This problem has been addressed through a strain of genetically modified rice. This rice produces the beta-carotene needed and will help to prevent the medical complications that arise when the body is denied this vital vitamin through

malnutrition (Miller, 2010). Genetic modification has also been used in the commercial sector for problems other than those revolving around diet. In Germany, for instance, a new type of potato has been engineered. This potato naturally manufactures a type of starch that can be used in the production of paper, clothing, and adhesive cement. These potatoes are not for consumption and will be used solely for commercial purposes, but the European Union has yet to approve of their existence on European soil (Rosenthal). This shows that even products that are not meant for human consumption, such as the aforementioned potatoes, are treated with distrust and have been a matter of contention between global powers for years. Genetic engineering has been taken out of the realm of science and has been placed firmly into the domain of politics. Therefore, it may be years before advancements in this field are made available to the world as a whole.

Escherichia coli

E. coli are gram negative facultative anaerobes (Todar, 2008). Facultative anaerobes have two metabolic pathways, one used in the presence of oxygen, and one used when oxygen is scarce. In this way, these bacteria can survive in a variety of environments. The pathway that is used in the presence of oxygen is known as respiration. In respiration, cells use oxygen and sugar (such as glucose) to produce energy and the waste product carbon dioxide. In fermentation, cells also produce energy, but oxygen is not used and alcohol, lactic acid, or other similar compounds are produced as well (Postlethwait, 2006). Since *E. coli* have both of these pathways, the bacteria can survive when shut into airtight containers. This property is of particular concern in the foodservice industry, since *E. coli* can be a potential health hazard in humans. There have been quite a few *E. coli* scares in recent history, the most memorable concerning spinach (Philpot).

Contrary to popular belief, however, most *E. coli* are not harmful to humans. In fact, there are billions of *E. coli* living in the human digestive tract at any given time. Beneficial *E. coli* are not limited to the inside of the body, however. Some of the most beneficial *E. coli* can be found in labs around the country, where scientists are striving to learn more about bacteria and find the answers to countless questions posed over the years about DNA. The genetic sequence of *E. coli* was the first to be completely mapped, making it an invaluable tool in the field of genetic research (Madigan, 2000). Even schoolchildren learn the basics of genetic science using *E. coli* as a model. Countless high school labs have teenagers working with this bacterium in order to gain hands on learning experience in DNA transformation.

Obligate Aerobes

Unlike *E. coli* obligate aerobic bacteria can only survive in the presence of oxygen. These bacteria lack the fermentative pathway that would enable survival in oxygen free environments. Unlike *E. coli*, obligate aerobes would die when placed inside of an airtight container. In order to make *E. coli* an obligate aerobe, the fermentative pathway would need to be either mutated beyond repair or blocked. It is necessary in this project for the *E. coli* used to be an obligate aerobe due to the fact that the respiration pathway must be used to produce the desired results.

Plasmids

Plasmids are small bundles of bacterial DNA that are not a part of the bacterium's natural genome. These bundles can be transferred from bacterium to bacterium through contact, and do not necessarily need to be passed from parent to daughters (Postlethwait, 2006). Many plasmids contain genetic codes that aid bacteria, such as antibiotic resistance or other modifications that

make them more fit to live in any given environment (Postlethwait, 2006). The plasmid pGLO, for instance carries a gene that makes the *E. coli* carrying it resistant to the antibiotic ampicillin. Other genes in a plasmid do not necessarily aid the bacterium in any way, but instead give it unique properties. One of the genes that fall into this latter category is the gene for which the pGLO plasmid gets its name, the GFP (green fluorescence protein) gene. This gene causes the bacteria that contain the pGLO plasmid to glow under ultraviolet light (see Figure 3). In no way does the glow help or hinder the *E. coli*, but it serves as a marker to show that the ampicillin gene is present (Miller, 2010). Plasmids are also the reason that "superbugs" are so hard to kill. Once a bacterium contains a plasmid that enables it to survive in conditions where most others would perish, it makes so many copies of this plasmid that it is virtually impossible to knock out. Not only does this tendency make that bacterium highly dangerous, but it will pass the same plasmid on to the surrounding bacteria, which will also make copies and become extremely difficult to kill. This is why the over prescription of antibiotics is so dangerous. The more often bacteria are exposed to antibiotics; the more likely they will develop resistance and pass that resistance on to the surrounding bacteria.

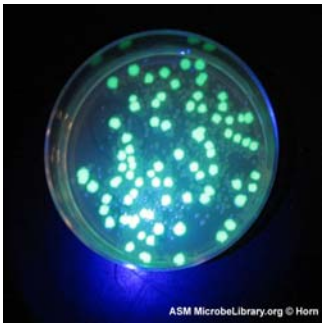


Figure 3: *E. coli* with the pGLO plasmid

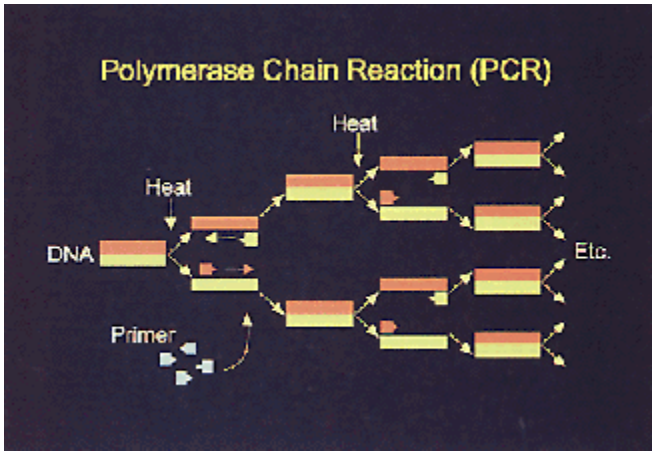
Genetically Engineered Plasmids

Plasmids do not only aid bacteria, but can also be genetically engineered so that the affected bacteria are able to aid humankind in some way. In recent years, scientists have been using plasmids to make bacteria more useful, such as pMicrodiesel (Kalscheuer, 2006). This plasmid enables *E. coli* to produce biodiesel- like fuel, which would help to alleviate humankind's reliance on petroleum products. Making biodiesel does not aid the bacteria in any way, but could potentially aid humans in the future. That is, once a way is found to enable the bacteria to produce enough biodiesel for commercial use. Currently, the yield of fuel from *E. coli* that contain the plasmid is far too low to be used, but it offers many prospects for the future. Plasmids such as pMicrodiesel could be the main technology of the future. Bacteria that can produce fuel, and a theoretically unlimited amount of bio-products as well, could effectively nullify the need for fossil fuels. That is still an idea for the future, since scientists still need to find ways of producing enough of whatever product is necessary to feed global demand. Once the technology is there, however, the bacteria-produced product should be much better for the environment, and potentially cheaper, than the fossil fuel equivalent.

PCR

One way to engineer plasmids is through the use of polymerase chain reaction (PCR), which is a way for scientists to make enough copies of a specific gene or genetic sequence to work with. It is a method that is much faster than using bacteria to clone DNA and uses a series of cycles to isolate and copy the target DNA (see Figure 3). The amplification of this DNA can aid in many areas, especially when engineering plasmids or testing DNA for various markers. It

utilizes polymerase, which is an enzyme that naturally copies and repairs DNA; to produce a great amount of the DNA needed for whatever the scientist that is using the technique desires. PCR has been used in many fields since its invention, not the least of which include molecular biology, healthcare, and forensic science. Scientists frequently use the PCR technique to produce a large amount of DNA so that it can be properly studied, especially when only a small amount of the desired gene is available. By making copies, the scientist ensures that he will be able to have enough of the desired DNA to meet his needs (Non- Hodgkin's Lymphoma Cyberfamily, 2009). This DNA can then be inserted into a plasmid, which can be transformed into bacteria. These bacteria will then take on the characteristic of that gene.



http://www.nhlcyberfamily.org/tests/pcr.htm

Figure 4: PCR

Materials and Methods

Materials

Chemicals/Combustibles	*Supplies*	*Equipment*
Phenol Red	HB 101 *E. coli*	Short wave UV light
	Plastic wrap	Incubator
	Micropipette	
	95 Well plate	
	Plastic loop	
	Shoe box	
	Sterile toothpicks	
	Lennox agar Petri dishes	

Procedure

HB 101 *E. coli* were plated on seven Lennox Agar Petri dishes, which were then grown inverted in an incubator at 37^{o}C overnight. After growth, colonies were then spotted on three plates. These plates were each placed into a modified shoebox individually. The shoebox was modified by cutting a 2X4in hole into the top. The plates of *E. coli* were then irradiated for 5 seconds, 6 seconds, and 7 seconds respectively with 254nm shortwave ultraviolet light and grown inverted overnight at 37^{o}C in an incubator. Phenol red medium was then placed into wells one through six A through H in three 96 well plates. Colonies were selected using a sterile toothpick for each and placed in wells (one well per colony). A control, *Bacillus thuringiensis,* was placed in well A1 for the purpose of comparison. Three sheets of oxygen- impermeable plastic wrap were stretched over the wells. The *E. coli* were grown overnight at 37^{o}C in an incubator. The colonies irradiated for 7 seconds were re- plated on another Lennox Agar Petri dish and irradiated once again for 7 seconds. These colonies were also grown according to the above procedure and placed into well plates and grown in an incubator at 37^{o}C.

Results

Table 1: Phenol Red Medium

Ingredient	Amount
Tryptone	10g
Yeast Extract	5g
NaCl	5g
Glucose	1g
Phenol red	25mg
Water(distilled)	1 liter

Note: This recipe was based on Lennox broth with the addition of phenol red for the purpose of indicating pH. If the broth showed that the solution was basic after the addition of the bacteria, the bacteria were obligate aerobes.

Table 2: pH Test

pH of Phenol red after	1 drop added	2 drops added	3 drops added	4 drops added
HCl	6	5	4	3
NaOH	8	9	10	11

Note: This test was conducted to ensure that the phenol red medium would accurately indicate pH.

Table 3: Mutation of Colonies after Irradiation

Number of seconds irradiated	Number of colonies retaining fermentative pathway	Number of colonies mutated
5	8	33
7	6	38
14	0	43

Note: This information was determined using the aforementioned color indication method.

Conclusion

The purpose of this experiment was to i) mutate *E. coli* to eliminate the fermentative pathway and ii) to develop a simple, novel phenol red indicator method to screen for the desired mutation. This was accomplished by streaking Lennox Agar Petri dishes with HB101 *E. coli*, which was then grown overnight inverted at 37^{o}C. The colonies isolated by this method were then spot plated and irradiated for 5 seconds, 6 seconds, and 7 seconds respectively. After growing these irradiated plates, the colonies were transferred to 96 well plates filled with phenol red medium. This medium had been developed prior to irradiation and was proven to be an able pH indicator through a pH test involving 0.1M HCl and 0.1M NaOH. Once the well plates were grown overnight, it was found that the colonies mutated at a rate of 89%. The colonies that mutated turned pink in the phenol red medium whereas the colonies that did not mutate turned orange.

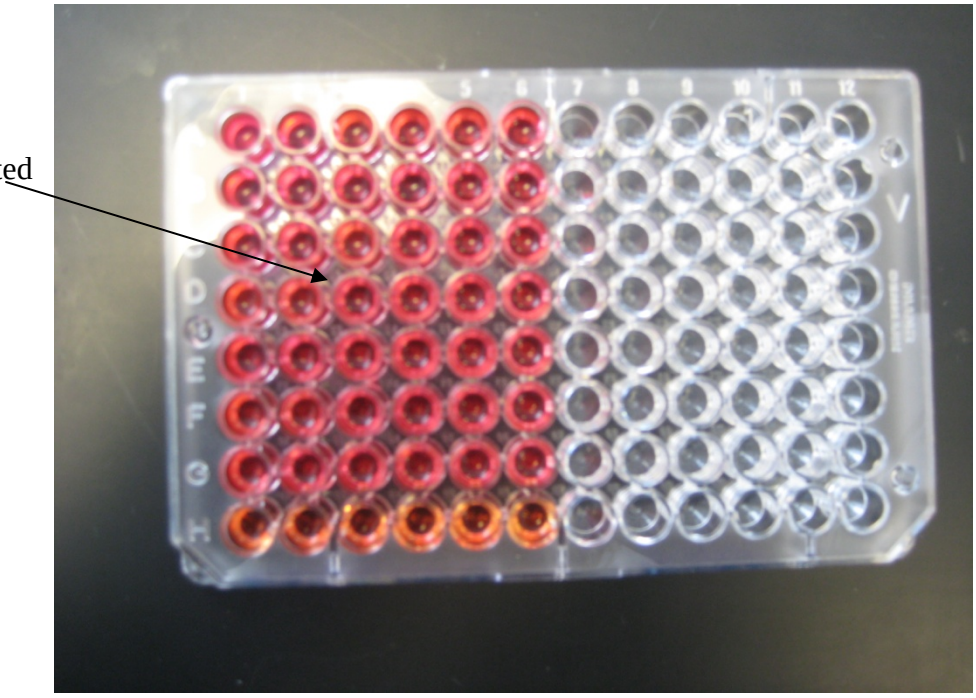


Figure 5: Mutated *E. coli*

The phenol red indication method is useful in determining whether or not the cells are fermenting, however it does not definitively determine the nature of the mutation, as it is possible that the colonies are not growing due to the fact that they are nutritional mutants rather than obligate aerobes. If the bacteria are nutritional mutants, adding various dietary supplements to the medium which the bacteria are grown on will allow growth with or without oxygen. More trials will need to be run to select the mutant best suited for the task in phase two. These trials will also add validity to the results, since it is possible that extenuating circumstances (such as nutritional mutation) are responsible for the lack of fermentation, rather than mutation of the targeted pathway. The nature of the mutation will need to be determined in the future before the second phase of the project can commence, reducing carbon dioxide to methanol.

Once the nature of the mutation is established the mutant best suited for phase two will be selected. This mutant will be, ideally, an obligate aerobe which can grow on Lennox agar without nutritional supplements. A plasmid containing formate dehydrogenase, formaldehyde dehydrogenase, and alcohol dehydrogenase will be constructed. These genes will first be amplified using the PCR technique and, once isolated, will be closed into a plasmid containing antibiotic resistance. Once this has been accomplished, the plasmid will be transformed into the genome of the aforementioned mutant *E. coli.* The bacteria will then be plated on both Lennox agar and Lennox agar containing antibiotics. The *E. coli* which grow on the plate containing antibiotics have picked up the plasmid and therefore will be able to reduce carbon dioxide to methanol. Other future research includes formulating a methanol collection device and researching the possibility of reducing carbon dioxide to methanol using bacteria which are naturally obligate aerobes.

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