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Biodiesel Glycerol Waste Byproduct as Potential Feedstock for Production of Biofuel

and Classroom Experiments

FINAL REPORT

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DISCLAIMERS

Biodiesel Glycerol Waste Byproduct as Potential Feedstock for Production of Biofuel

ABSTRACT

In making biodiesel from plant and animal oils, glycerol is a byproduct. The biodiesel is burned in engines but the supply of glycerol, which is not a biofuel, exceeds the demand. It may be possible for some microbes that utilize biodiesel glycerol byproduct to make biofuel or other products. Selected microbes of interest to bioenergy research which were likely to utilize pure glycerol were assessed for ability to utilize glycerol byproduct as a carbon source. Samples inoculated on culture medium failed to produce evidence of any resident microbial flora. Growth of *Escherichia coli* bacteria in broth containing byproduct from manufacture of biodiesel by transesterification showed a decrease, while growth in culture containing byproduct made by catalyst column was slightly increased. Selected microorganisms which do not use glycerol were not able to find chemical components in byproduct for growth. *Escherichia coli,* and *Klebsiella* species, which may be genetically engineered to make biofuel and are known to utilize glycerol, showed more growth from byproduct from catalyst column than that from transeseterification. A positive test for presence of alcohol was shown by *E. coli* cultures grown on glucose, and glycerol. Stock solutions of glycerol byproduct themselves showed a positive test for the presence of alcohol. *Streptomyces* species, which have been suggested as a source of lipids, showed growth on media containing byproduct. Drops of the culture eluent from the antibiotic synthesizing *Streptomyces griseus* inhibited growth of test bacteria. *Clostridium acetobutylicum*, which makes butanol, did grow in presence of added byproduct. However, byproduct in medium did not appear to increase growth. Glucose had a significant effect on increasing growth and alcohol was detected in cultures containing glucose as the single added carbon source. Samples of heat killed, capsule producing *K. pneumoniae* used as a carbon source appeared to support some growth of selected carbohydrate utilizing microorganisms. This work has helped to identify microorganisms that could utilize glycerol byproduct as an industrial feedstock and provided topics for student projects dealing with biofuel technology. Undergraduate students assisting with the project received a valuable educational experience in laboratory investigations.

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1. Background

1.1. Biodiesel

The principal chemical component of biodiesel is fatty acid methyl esters. Biodiesel is commonly made by transesterification, which is process of alkaline hydrolysis of biological oil by using methanol, sodium hydroxide and heat (www.biodiesel.org ; www.biomich.com; Kalscheuer et al, 2006). The main sources are soybean oil and animal grease. Vegetable oils and animal fats provide triacylglycerols (TAGs). The TAGs in oil are the source of fatty acids. Triglyceride oil is broken to methyl esters (biodiesel) and glycerol byproduct. Three liters of biodiesel are produced for every liter of glycerol byproduct. Glycerol is not a biofuel but it may be possible for some microorganisms that utilize glycerol to make biofuel or other products as well as help solve a waste disposal problem. A site map exists for functioning or planned biodiesel plants (www.michigan.gov/mda).

1.2. Butanol

Butanol is a four carbon alcohol that can be used in production of important products such as plasticizers, lacquers and resins. Butanol currently has value for potential use as fuel extender or fuel (www.butanol.com). Butanol has a lower volatility, lower hydrophobicity, higher miscibility with other hydrocarbons and higher energy content than ethanol. Acetone-butanol-ethanol (ABE) microbial fermentation was the main source of butanol and acetone until the early 1950s when it became more cost effective to produce these chemicals from the petroleum industry. However, the supply of petroleum is expected to decrease. As the price of gas from petroleum goes up, butanol may be made by the microbial fermentation industry again. This would be good for farmers because the basic substrate (feedstock) for butanol is starch (grains). Butanol is made by the bacterium known as *Clostridium acetobutylicum*. It is an anaerobe, which means that it can only grow in the absence of oxygen or it will be killed. Consequently, special environmental conditions are required for culture. Synthesis of product by the organism has two phases (Jones and Woods, 1986). (1.) In acidogenesis there is vegetative growth where organic acids acetate and butyrate are produced. (2.) In solventogenesis, acids produced are transformed into acetone, butanol and ethanol. When some microbes become old, they make spores.

It has been reported that sporylation is necessary for solvent production (Woods, 1995). Recently, a mutant of *Clostridium beijerinckii* has been reported that produces an increased level of butanol in a bioreactor (www.theengineer.co.uk/Articles/312768/).

1.3. Ethanol

Ethanol is a two carbon alcohol that is used in gasahol to reduce automotive emissions and reduce dependence on petroleum oil (www.eere.energy.gov; www.ethanol-gec.org).The production of ethanol is a benefit for farmers because it is made by microbial fermentation by the yeast known as *Saccharomyces cerevisiae* which uses agricultural products as substrates. Output of fuel ethanol from corn has tripled from 2000 to 2006 and is still rising (www.ethanolrfa.org). Unfortunately, it is not possible to produce enough ethanol from corn to satisfy gas demand.

To produce ethanol from grain, starch must be converted to sugar for the yeast. Starch is heated in water to make hydrogen bonds weak. Added amylase enzymes subsequently break starch chains into small pieces and glucose.

Inside the cell, one glucose molecule is converted to two ethanol and two CO_2s (Russell, 2003). *Saccharomyces cerevisiae* does not ferment some other sugars (xylose and arabinose). Genetically engineered *S. cerevisiae* can ferment xylose to ethanol (Kotter et al, 1990). Genetically engineered microbes, which are not part of nature, can be unstable (do not perpetually function), are controlled by the companies that hold the patents, and their use make some people uncomfortable. Because it is not a food crop, lignocellulose biomass (stems, wood, paper products and grass) has become of interest as a feedstock for yeast. Basic chemical components include cellulose, hemicellulose and lignin (Wyman, 1994). However, ethanol producing yeast will not utilize these substances. Consequently, chemical methods (acid, alkali, hot water and organic solvents have been devised to break down the indigestible polymers into subunits (Saha, 2004). Dilute acid is commonly used but chemicals toxic to yeast (acetic acid, alcohols, aromatic compounds, furfural and hydroxymethylfurfural) are produced (Luo, 2002). By a biological means, three different enzymes are needed to break cellulose down to glucose. Overall, conversion of lignocellulose is currently not cost effective.

2. Vision

The glycerol waste product from biodiesel plants might eventually be used to make more biofuel or other products. Laboratory exercises will help educate students about biodiesel and finding applications for waste from manufacturing.

3. Description and Accomplishments

3.1. Byproduct Appearance and Normal Flora/Sterility

In contrast to pure glycerol and byproduct made from a catalyst column, byproduct from manufacture of biodiesel by transesterification was pale yellow and pasty (Fig. 1). The byproducts did not appear to be the same material as glycerol. Other investigators are seeking to research applications for biodiesel glycerol byproduct with the assumption that it is "glycerol" (Murarka et al., 2008).

Many industrial products naturally harbor microbes. Samples (.1 ml) of unsterilized byproduct made by transesterification or catalyst column which were inoculated on rich medium - brain heart infusion (BHI) agar, which supports a variety of microorganisms, and incubated at 25^{o}C for 7 days failed to produce evidence of any resident microbial flora. This suggested that there might not be a need for sterilization of byproduct in some applications in industrial microbiology. Standard media used to grow cultures to make products needs to be sterilized which adds expense to the process.

3.2. Toxic Substances and Nutrients for Microbes

As mentioned above, lignocellulose treatment with dilute acid causes the formation of toxic products such as furfural. Biodiesel glycerol product may also contain chemicals that are toxic to microbes. In preliminary work, byproduct made by transesterification or catalyst column did not produce any bacterial growth inhibition zones when incubated at 37^{o}C for 24 h on a lawn of *Escherichia coli* inoculated on BHI agar (Fig. 2).

For a quantitative test, bacteria were grown in nutrient broth (NB) to which glycerol byproduct had been added and the growth compared with that produced in broth without the byproduct (Fig. 3). Growth in culture containing byproduct from transesterification was decreased while growth in culture containing byproduct from catalyst column was slightly increased. Nutrient broth is a general purpose liquid culture medium. Growth was assessed quantitatively in this and other experiments by measuring culture optical density (OD) in a Klett – Summerson colorimeter.

Before pH adjustment for use in media a 20% stock solution of byproduct made from transesterification showed high alkaline pH, in contrast to that for byproduct from the catalyst column or pure glycerol which was slightly acidic (Table 1). The high alkaline pH observed with the byproduct from transesterification was likely to be due to the sodium hydroxide used in the manufacturing process. For remaining project experiments, solutions of glycerol byproduct needed pH adjustment before use in culture media.

Glycerol byproduct may contain other nutrient chemicals besides glycerol. In phenol red broth, which is a differential medium that assesses the ability of an organism to ferment an added carbon source, *Acinetobacter calcoaceticus, Alcaligenes faecalis,* and *S. cerevisiae* which do not use glycerol did not produce acid from byproduct made by transesterification or catalyst column (Table 2). Production of acid indicated use of the carbon source (Fig. 4). On minimal agar, containing only the essential nutrients for wild type strains and byproduct as a potential carbon source, *A. calcoaceticus*, *A. faecalis,* and *S. cerevisiae* did not produce growth (Table 3; Fig. 5). Inability of these microorganisms to produce a positive reaction in these tests suggested that byproduct did not contain a variety of carbon sources to support growth. *Pseudomonas* species were screened in an attempt to find a strain that did not ferment glycerol. Minimal agar medium was prepared as described by Miller (1972) except that sodium chloride was substituted for sodium citrate because some microbes utilize citrate as a carbon source. Phenol red broth and growth on minimal agar are qualitative tests.

3.3. Host Bacteria for Biofuel Synthesis

Escherichia coli and *Klebsiella* species have become important in biofuel research because strains (containing foreign genes) have been constructed by genetic engineering that produce ethanol (Ingram et al., 1991; Ohta et al., 1991). Moreover, *E. coli* has recently been genetically engineered to produce butanol and biodiesel respectively (Connor and Liao, 2008; Kalscheuer et al., 2006). These bacteria are known to naturally utilize pure glycerol as a carbon source. Consequently, representatives of these species were screened for their ability to utilize glycerol byproduct made by transesterification or catalyst column. In phenol red broth, *E. coli,* and

Klebsiella pneumoniae demonstrated a positive reaction (produced acid from fermentation) in presence of byproduct (Table 2). On minimal agar, wild type *E. coli* and *K. pneumoniae*, produced visible growth in presence of byproduct (Table 3). In an attempt to obtain quantitative data, these microbes were grown in minimal broth that contained only the byproduct as a carbon source (Fig. 6; Fig. 7). There was not a considerable difference between culture ODs produced from glycerol and byproduct from catalyst column.

Byproduct from transesterification did not support growth as well as that from catalyst column. *K. pneumoniae* appeared to use glycerol and byproduct better than *E. coli* K12. Both organisms grew significantly better on glucose than pure glycerol. A rapid solid phase chemistry enzyme (glucose oxidase and peroxidase) based assay (Precision Labs, Inc.) was used to detect glucose in cultures after incubation. Both *E. coli* and *K. pneumoniae* lowered added glucose to an undetectable level (Table 4). A rapid solid phase chemistry enzyme (alcohol oxidase and peroxidase) based assay (AlcoScreen) was used to detect alcohol. The manufacturer suggested that the test can score for alcohols having up to 4 carbons and will not react with glycerol. Interestingly, *E. coli* showed a positive test for the presence of alcohol in cultures produced from glucose and glycerol (Table 4). Unfortunately, it was discovered that stock solutions of glycerol byproduct made by transesterification and catalyst column showed a positive test for the presence of alcohol. Therefore, it was not possible to determine if any alcohol may have been produced in cultures containing those carbon sources.

$$CH_3CH_2OH \xrightarrow{\text{Alcohol Oxidase}} CH_3\overset{H}{C} = 0 + H_2O_2$$

$$H_20_2 + DH_2 \xrightarrow{\text{Peroxidase}} D\ (\text{Blue}) + 2H_20$$

Alcohol dectection reactions

Production of alcohol is considered to be a fermentative process. Cultures grown here were incubated with exposure to air. *Escherichia coli* and *K. pneumoniae* can grow aerobically or anaerobically – they are facultative. It is possible that anaerobic incubation could alter the results. Most recently, increased alcohol production has been described in mutated *E. coli* (Trinh and Srienc, 2009). The pH was determined by spotting a small sample of culture on pH paper and was in the range of 7 – 8. Use of pH paper permitted the use of a small sample and avoided contamination of a culture with a probe.

3.4. Natural Lipids for Biodiesel

Fatty acids for making biodiesel come from triacylglycerols found in vegetable oil or animal fat. Some microbes such as *Streptomyces* and its relatives naturally contain high levels of triacylglycerols (Olukoshi and Packter, 1994). These microbes may be a prospective source of fatty acids for biodiesel. However, a process to make biodiesel from these microbes has not been developed. There would be a need for an inexpensive method to grow the cells that contain triacylglycerols. A standard complex medium (actinomycete agar) for these microbes does use pure glycerol as an ingredient (Difco Laboratories, 1985). In this study, *Streptomyces albus* produced growth on minimal agar containing byproduct from transesterification or catalyst column (Table 3). (This microbe grows poorly in liquid medium)

To obtain quantitative data, a means to grow *Streptomyces* in submerged - broth culture on byproduct needs to be devised.) *Streptomyces* species are known as important organisms for making products in industrial microbiology. *Streptomyces* species have been reported to show cellulase activity, which is caused by a type of enzyme used to make glucose for yeast (MacKenzie et al., 1987). It might be profitable to find applications for these microorganisms grown on byproduct.

Interestingly, a pilot experiment was performed to attempt production of antibiotic from *Streptomyces griseus* grown on medium containing glycerol byproduct as a nutrient. *S. griseus* is known for production of streptomycin. The organism was grown on actinomycete agar and minimal agar containing byproduct from catalyst column instead of glycerol – the regular ingredient (Fig. 8). Antibiotic was recovered from plate cultures by elution into normal saline. The first few drops of saline formed beads on the surface of the microbial mat. The culture mat appeared to be hydrophobic. It is tempting to suggest that this is associated with the high lipid content of cell. After filter sterilization, drops of eluent were assessed for antibiotic activity by development of bacterial growth inhibition zones (clear areas) on a lawn of *E. coli* inoculated on BHI agar (Fig. 9). Although eluent from AA and minimal agar both produced bacterial growth inhibition zones, the one made from AA appeared to be more potent. This could be due to the provision of extra growth factors.

3.5. Feedstock for Biobutanol

Clostridium acetobutylicum is known to produce butanol but the standard feed stock in industry is corn meal mash. Reports existed that at least some strains may utilize glycerol (Girbal and Soucaille, 1994; Vasconcelos et al., 1994). The ability of the organism to utilize glycerol byproduct was investigated here by attempting to grow the organism in the classic fluid thioglycolate medium (FTM) but with glycerol byproduct as an added carbon source (Difco Laboratories, 1985). Glucose was not included in the base medium (FTM without glucose) so that the utilization of added carbon sources could be examined. A characteristic use of FTM is the cultivation of some of the less strict anaerobic bacteria such as clostridia. Sodium thioglycolate decreases the oxidation reduction potential (Eh) to assist growth of anaerobic bacteria. The more recent Oxyrase For Broth (Oxyrase, Inc.) enzyme system was used in FTM to help create anaerobiosis. Special commercially prepared enzymes in this supplement protect the anaerobic bacteria from oxygen toxicity.

Nevertheless, growth of the organism was very slow compared to other bacteria. In experiments, *C. acetobutylicum* did grow in presence of added byproduct made from transesterification or catalyst column. However, presence of byproduct in medium did not appear to increase growth of *C. acetobutylicum* (Fig. 10). Turbidity at top of some cultures suggested a little use of byproduct but there was no enhancement of OD (Fig. 10). Glucose had a significant effect on increasing growth. In presence of glucose, the pH of medium would drop from 7 – 8 to 5. When the presence of glucose was assessed by the glucose oxidase and peroxidase based assay after incubation, the concentration could drop to an undetectable level. The alcohol oxidase and peroxidase based assay used to detect alcohol suggested the production of alcohol in cultures containing glucose as the single added carbon source.

Although cultures containing glycerol byproduct tested positive for alcohol content, the meaning is unclear because glycerol byproduct made by transesterification and catalyst column showed a positive test for the presence of alcohol. The strain of *C. acetobutylicum* used in this study did not exhibit any appreciable use of glycerol byproduct or glycerol.

This investigation was inspired by the report that production of butanol by *C. acetobutylicum* from glucose was increased in the presence of glycerol (Vasconcelos et al., 1994). A variety of explanations for this observation could exist. The strain used by those investigators may have been unique. Glycerol may have acted as a steering agent to increase butanol production but not growth in the presence of glucose. The high concentrations of glycerol used in the medium by those investigators may not have provided a carbon source but have increased the medium viscosity to create a desirable environment for growth and production of butanol. Some microorganisms will not grow well in a watery medium. The FTM contains a small amount of agar as thickener. In nature, clostridia are normally found in viscous sediments and mud.

3.6. Carbohydrates for Bioethanol

Some microbes that are able to utilize pure glycerol as a carbon source may produce a carbohydrate covering around the cell called a capsule. It can be quite large in comparison to the size of the cell. Some of these capsules consist of dextran which is a polymer of glucose. It might be possible to break down the capsule to provide glucose for ethanol producing *S. cerevisiae*. There is an old precedent for this type of idea. Starch from plants is commonly broken down by an added enzyme (amylase) to produce glucose for ethanol production by yeast. Microbes producing capsules (such as *Klebsiella* species) would be grown in medium containing glycerol byproduct as the carbon source, they would be harvested, treated with digestive enzymes (dextranase and lysozyme) and used as the potential carbon source for yeast. Dextranase is an enzyme that breaks down dextran and lysozyme is an enzyme that breaks open cells by damaging the peptidoglycan.

In a preliminary experiment, samples of heat killed *K. pneumoniae* used as the carbon source on minimal agar medium appeared to support some growth of *K. pneumoniae*, *E. coli* K12, *S. cerevisiae* and *S. albus.* This may not be surprising since microbiological media frequently contains components prepared from cells of other organisms – especially yeast (Difco Laboratories 1985).

In a separate experiment, dextranase (Sigma) plus dextran plus *S. cerevisiae* in phenol red fermentation broth showed a positive (acid) reaction. This suggested that the enzyme digested the dextran to sugar which was used by the yeast. However, assessment of the culture and enzyme stock bottle showed that the dextranase was contaminated with a dextran degrading microorganism. Filter sterilization of the enzyme product removed the ability to degrade dextran. Consequently, dextranase product purchased was not suitable for doing the primary experiment. A different commercial dextranase might be more effective. Despite complications with the commercial dextranase, it may still be feasible to grow microbes on glycerol byproduct and use these as feedstock for other microbes. Preliminary research did indicate that *K. pneumoniae* utlized glycerol byproduct made by transesterification or catalyst column.

4. Evaluation

The results from experiments described above were used to design laboratory projects for students. Moreover, because microorganisms being investigated could utilize glycerol byproduct, there is potential for development.

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6. List of Tables

Table 1. The pH of Byproduct Solutions

	Source of glycerol or byproduct		
Liquid	Pure Glycerol	Byproduct from Transesterification	Byproduct from column
20% stock	3.7	12.2	5.1
0.2% solution	4.9	9.8	5.9
0.2% in phenol red broth	7.3	7.4	7.3

Note: Plain phenol red fermentation broth demonstrated pH 7.4.
Byproduct from transesterification was pH adjusted before use in culture media.

Table 2. Fermentation of Glycerol Byproduct in Phenol Red Broth

	Carbon source			
Microorganism	None added	Pure Glycerol	Byproduct from Transesterification	Byproduct from column
A.calcoaceticus	(-)	(-)	(-)	(-)
A. faecalis	(-)	(-)	(-)	(-)
E. coli K12 auxotroph	(-)	(+)	(+)	(+)
K. pneumoniae	(-)	(+)	(+)	(+)
Pseudomonas	(-)	(-)	(-)	(-)
S. albus	(-)	(-)	(-)	(-)
S. cerevisiae	(-)	(-)	(-)	(-)

Note: (-), Nonfermentation of carbon source (red); (+), fermentation of carbon source (yellow).*Pseudomonas* species included *P. aeruginosa*, *P. fluorescens*, and *P. putida*.
S. cerevisiae gave a positive reaction when glucose served as a carbon source.
Concentration of added carbon source was .2%. Auxotroph, cannot grow unless select nutrients are provided. Incubation at 25^{o}C for 48 hrs.

Table 3. Growth of Microorganisms on Minimal Agar Containing Glycerol Byproduct

	Carbon Source				
Microorganism	None Added	Glucose	Pure Glycerol	Byproduct from Transesterification	Byproduct from column
A. calcoaceticus	(-)	(-)	(-)	(-)	(-)
A. faecalis	(-)	(-)	(-)	(-)	(-)
E. coli B	(-)	(+)	(+)	(+)	(+)
E. coli K12 auxotroph	(-)	(-)	(-)	(-)	(-)
K. pneumoniae	(-)	(+)	(+)	(+)	(+)
Pseudomonas	(-)	(+)	(-)	(-)	(-)
S. albus	(-)	(+)	(-)	(-)	(-)
S. cerevisiae	(-)	(+)	(-)	(-)	(-)

Note: (+), Growth of streak plate culture; (-), no growth of streak plate culture.
Vitamin B1 added to all plates for *E. coli* K12 (Miller, 1972).
Auxotroph, can not grow unless select nutrients are provided.
Pseudomonas species included *P. aeruginosa*, *P. fluorescens*, and *P. putida*.
Incubation at 25°C for 7 days.

Table 4. Glucose and Alcohol in Minimal Medium Cultures

		Glucose		Alcohol	
Strain	Carbon source	Control	Experimental	Control	Experimental
E. coli K12	Glucose	(+)	(-)	(-)	(+)
	Pure glycerol	(-)	(-)	(-)	(+)
K. pneumoniae	Glucose	(+)	(-)	(-)	(-)
	Pure glycerol	(-)	(-)	(-)	(-)

Note: Control, uninoculated minimal medium.
Experimental, culture in minimal medium.
(+), Test substrate present. (-), Test substrate absent.
The pH of cultures was 7 – 8. Incubation at 25°C for 2 weeks.

7. List of Figures

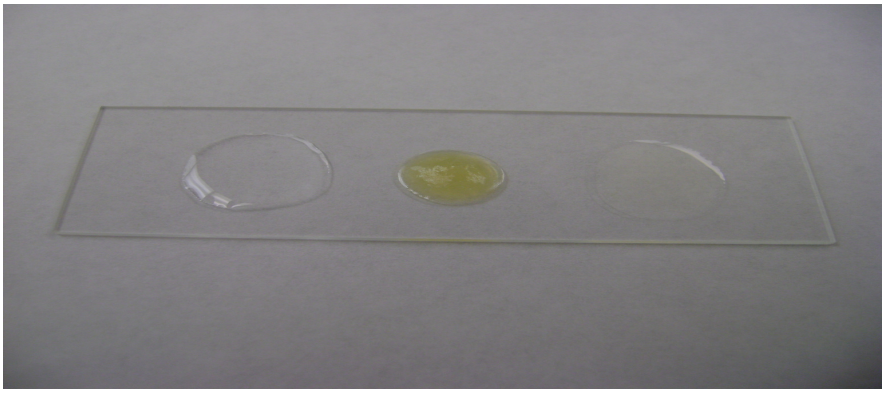

Figure 1. Glycerol Byproduct from Manufacture of Biodiesel
Left, pure glycerol; center, glycerol byproduct from transesterification (Power Alternative); right, glycerol byproduct from catalyst column (Next Energy).
Sample size = 50 µl.
Glycerol byproduct from transesterification was likely to contain soap.
Samples of byproduct inoculated on BHI agar and incubated at 25°C for 7 days failed to produce colonies of microorganisms.

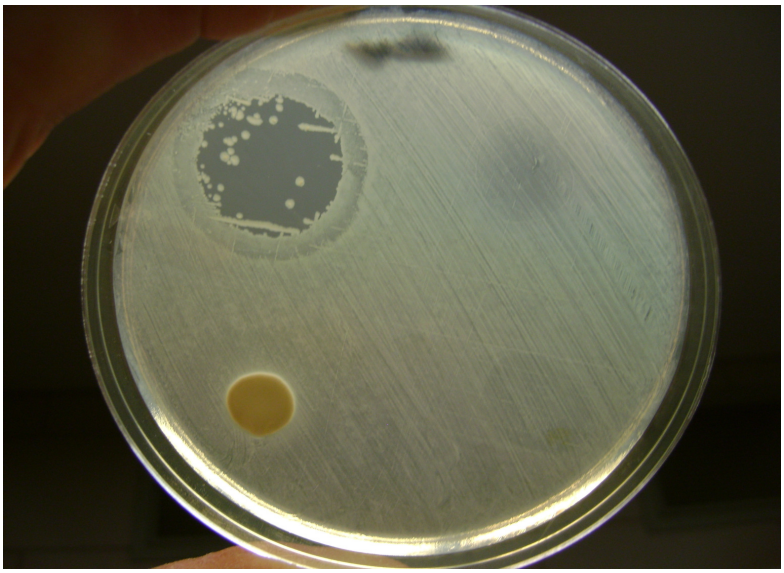

Figure 2. Toxicity of Glycerol Byproduct to Microorganisms
Top left, ethanol (positive control); top right, pure glycerol; bottom left, glycerol byproduct from transesterification; bottom right, glycerol byproduct from catalyst column. Sample size = 50 µl. Test microorganism was *E. coli* K12 on BHI agar. Incubation was at 37°C for 24 hrs.

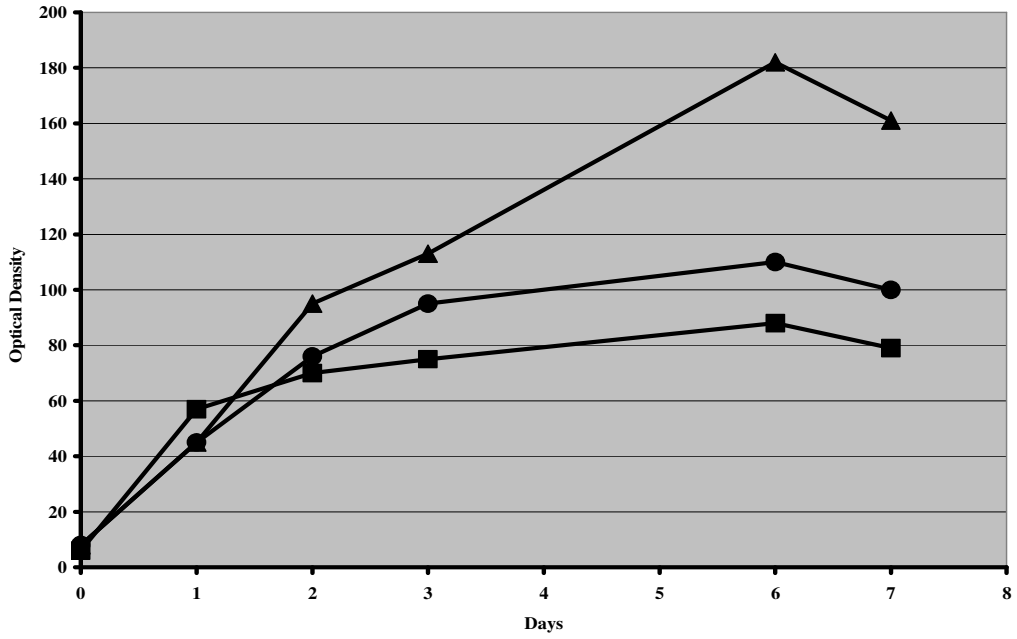

Figure 3. Growth of *E. coli* in Nutrient Broth Containing Glycerol Byproduct
Circle, no byproduct added; square, byproduct from transesterification; triangle, byproduct from column.
Concentration of sterilized byproduct added was .2%.
Byproduct was not pH adjusted.
Inoculum cells grown on nutrient agar.
Incubation at 25°C.

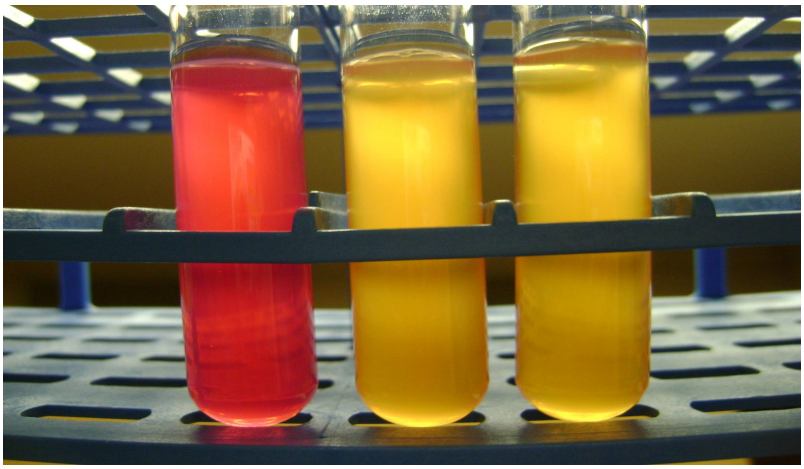

Figure 4. Fermentation of Glycerol Byproduct in Phenol Red Broth by *E. coli* K12
Left, no carbon source; center, pure glycerol; right, biodiesel glycerol byproduct from catalyst column. Incubation at 37°C for 18 hrs.

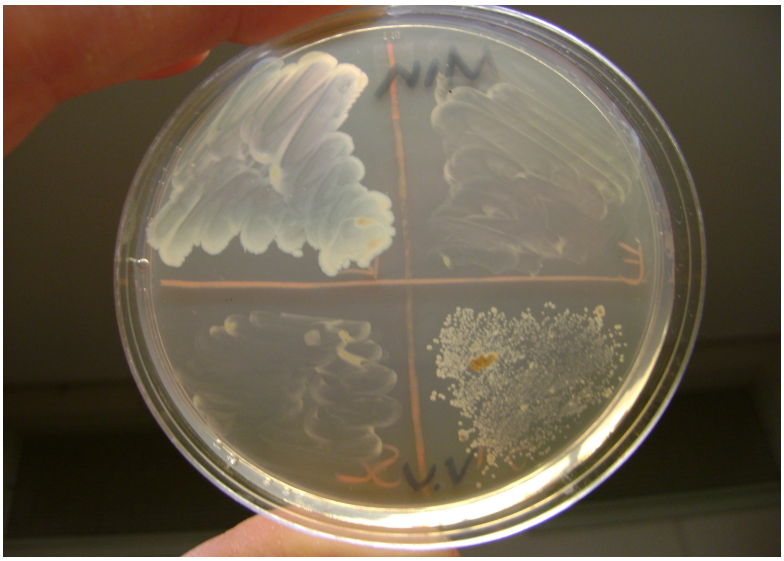

Figure 5. Growth of Microorganisms on Minimal Agar with Glycerol Byproduct from Catalyst Column

Upper left, *K. pneumoniae*; upper right, *E. coli* K12 auxotroph; lower left, *S. cerevisiae*; lower right, *S. albus.*

Auxotroph, can not grow unless select nutrients are provided.

Incubation at 25^{o}C 7 days.

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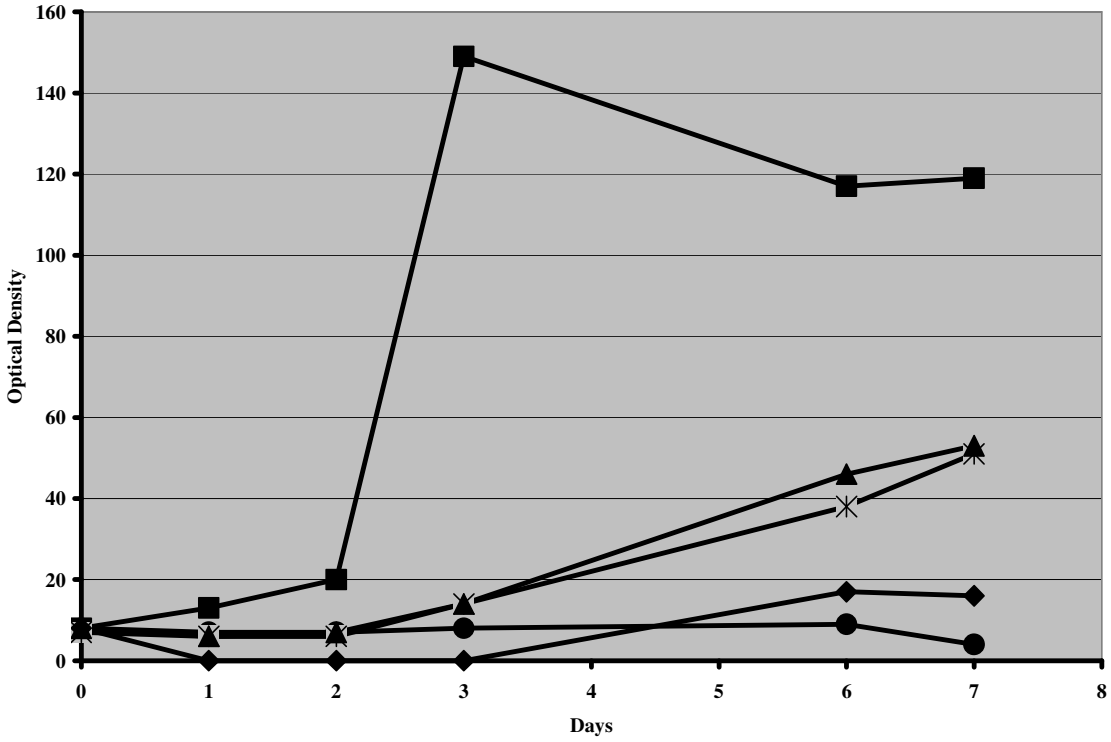

Figure 6. Growth of *E. coli* K12 in Minimal Broth Containing Glycerol Byproduct
Circle, no carbon source; square, glucose; triangle, glycerol; diamond, byproduct from transesterification; star, byproduct from column. Concentration of carbon source was .2%. Byproduct was pH adjusted and sterilized. Incubation at 25°C.

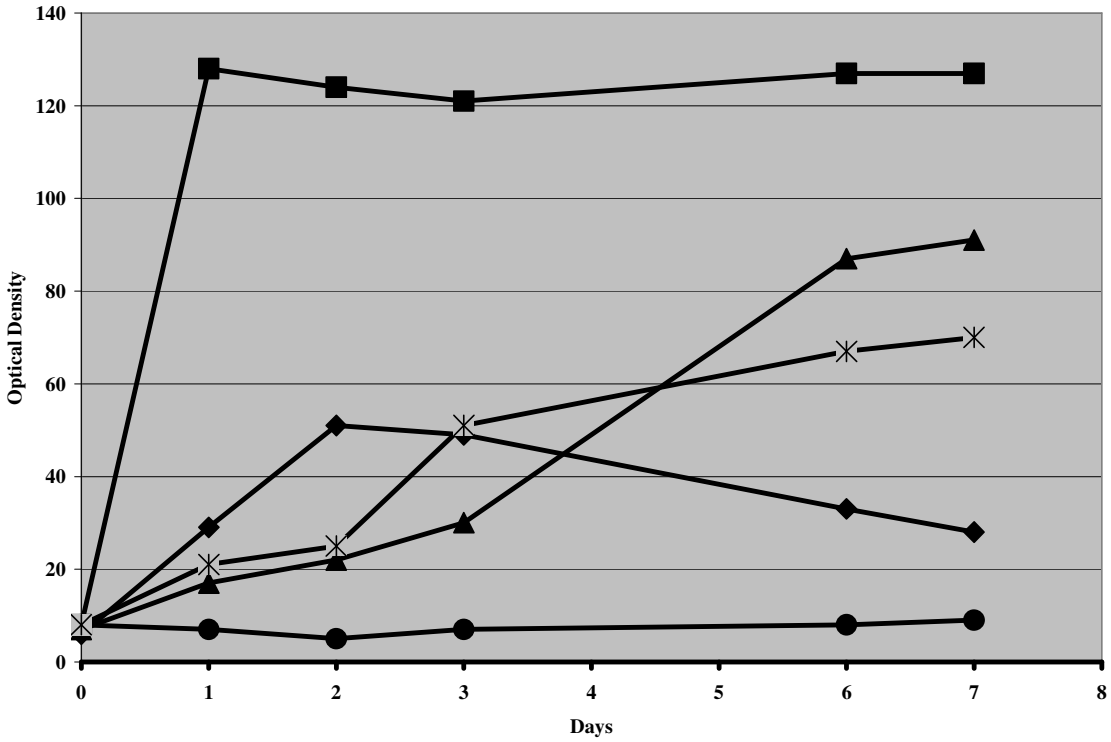

Figure 7. Growth of *K. pneumoniae* in Minimal Broth Containing Glycerol Byproduct
Circle, no carbon source; square, glucose; triangle, glycerol; diamond, byproduct from transesterification; star, byproduct from column.
Contentration of carbon source was .2%.
Byproduct was pH adjusted and sterilized.
Incubation at 25^{o}C.

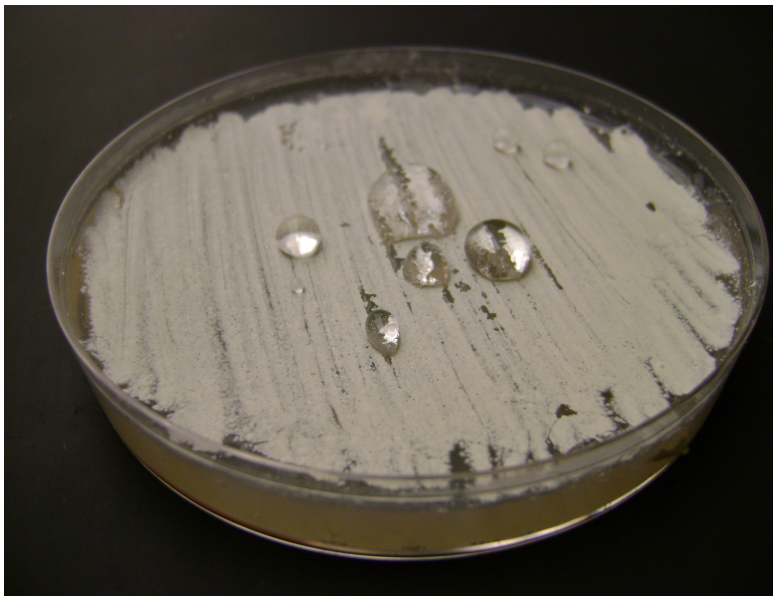

Figure 8. Drops of Normal Saline on Hydrophobic Culture Mat of *S. griseus*
Inoculation was performed with a swab.
Actinomycete agar contained .5% byproduct.
Incubation at 25°C for 2 weeks.
Plate was flooded with 10ml saline and stored at 4°C for 48 h before collection of eluent.

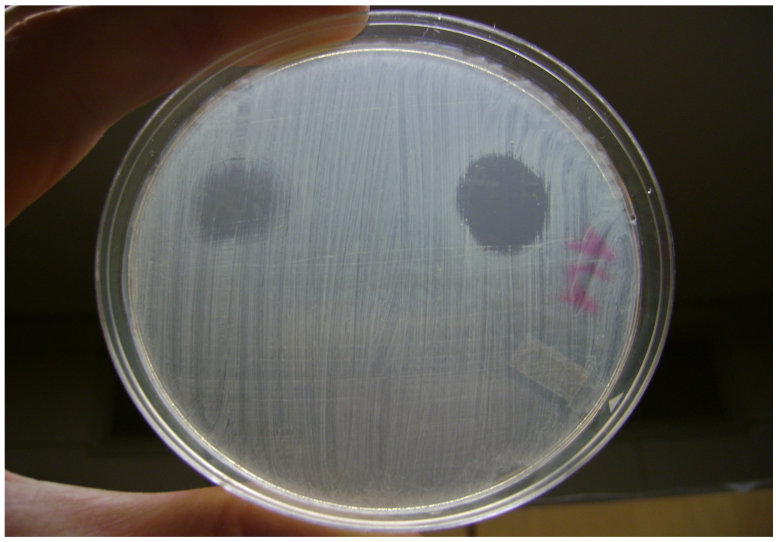

Figure 9. Antibiotic made by use of Biodiesel Glycerol Byproduct from Catalyst Column
Top left, eluent from minimal agar culture; top right, eluent from actinomycete agar culture; bottom center, normal saline (control).
Sample size = 50 µl.
Test microorganism was *E. coli* K12 on BHI agar.
Incubation was at 37°C for 48 hrs.

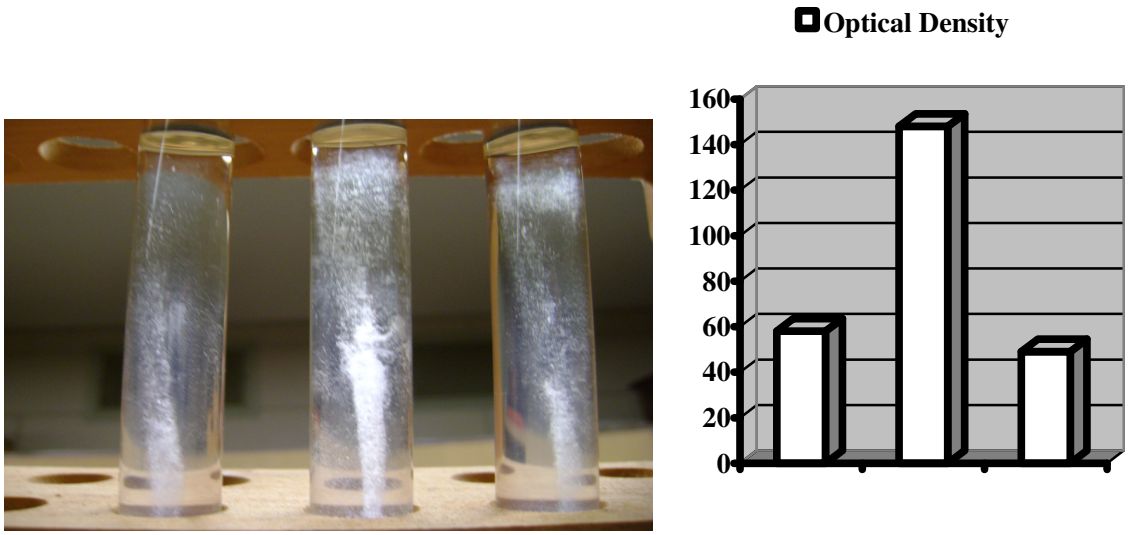

Figure 10. *C. acetobutylicum* Cultured in Fluid Thioglycolate Medium containing Glycerol Byproduct

Left, no added carbon source; center, glucose; right, glycerol byproduct from catalyst column.
Base medium was fluid thioglycolate medium without glucose.
Oxyrase reagent was added to all tubes.
Contentration of added carbon source was .2%.
Byproduct was pH adjusted and sterilized.
Inoculum was grown in fluid thioglycolate medium and diluted 1/100.
Incubation at 25^{o}C for 4 weeks.
Cultures were vortexed after incubation before assessment of optical density.

Experiment #1: BIOFUEL PRODUCING MICROORGANISMS UNDER THE MICROSCOPE

Purpose: this exercise is for students to observe the cell shape, arrangement and size of some microorganisms that can be used for biofuel research.

Background: Microorganisms of interest to biofuel research exhibit a variety of cell shapes, arrangements and sizes. Many of these microorganisms are bacteria (prokaryotes). *Escherichia coli* and *Klebsiella* species, which have been used as hosts to genetically engineer biofuel making cells, are rods that usually exist as singles or pairs. *Clostridium acetobutylicum* (an anaerobe) which is a natural producer of butanol also exists as rods that are usually found as singles or pairs. *Streptomyces* species, which have been suggested as a possible source of lipids for biodiesel, exists as rods in long chains. *Saccharomyces cerevisiae*, a yeast type of fungus has an egg-shaped cell. It is a type of cell that is large and more complex (eukaryote). However, a smaller cell has a greater surface to volume ratio and works as more efficient chemical factories. Cells are fairly transparent, so the easiest way to observe how they appear is to color them by a technique known as the "simple stain" before microscope examination.

Items to know:

- Liquid biofuels: ethanol, butanol, and biodiesel.
- Ethanol: a two carbon alcohol that is used in gasahol to reduce automotive emissions and reduce dependence on petroleum oil (www.ethanolrfa.org).
- Butanol: a four carbon alcohol that can be used in production of important products such as plasticizers, lacquers and resins. Butanol currently has value for potential use as fuel extender or fuel (www.butanol.com).
- Biodiesel: the principal chemical component is fatty acid methyl esters. Biodiesel is commonly made by transesterification, which is process of alkaline hydrolysis of biological oil by using methanol, sodium hydroxide and heat (www.biodiesel.org; www.biomich.com.).
- Anaerobe: a microorganism that can thrive in the absence of oxygen and is killed by oxygen.
- Simple stain: a procedure to color microbes with only one dye so that they may be more easily observed.

- Stain or dye: an organic compound with a chromophore group (gives color) and a auxochrome group (gives a positive or negative charge).

- Morphology: bacillus (rod), coccus (sphere), yeast (egg-shaped cell).

- Configuration: singles, pairs, tetrads, clusters, or chains.

Materials:

1 of each culture per pair/table:

Escherichia coli (37°C nutrient broth culture)
Klebsiella pneumoniae (37°C nutrient broth culture)
Clostridium acetobutylicum (25°C fluid thioglycolate broth)
Streptomyces albus (25°C nutrient agar slant)
Saccharomyces cerevisiae (37°C Sabouraud dextrose agar slant)

Cultures can be purchased directly from a biological supply house or grown fresh with standard culture media.

1 Compound microscope
1 Set of stains: crystal violet (purple) and safranin (red)
1 Box of glass slides
1 Wax pencil/marker
1 Staining rack
1 Pad of bibulous paper
1 Pad of lense paper
1 Bottle of immersion oil
1 Inoculating loop
1 Bunsen burner
1 Slide discard container
1 Distilled water bottle

Methods:

1.) With a marker draw a circle on one side of a glass slide. On the flip side spread 2-6 loopfuls of broth culture inside the borders of the circle (Fig.). In order to prepare a smear of microorganisms that were grown on an agar slant, a drop of water is placed on a slide in the borders of the circle and a very small amount of the culture recovered with an inoculating loop is gently mixed in. It is important to be careful not to make a paste where the cells would be to close together.

2.) Permit the samples to air dry or place the slides in the incubator to encourage evaporation. "Heat-fix" the bacteria to the slide by passing the dried sample face up through the flame of a gas burner for two or three seconds. Do not cook the sample! Place the slide face up on a staining rack. Flood the sample with crystal violet or safranin and wait one minute. Rinse the slide gently with tap water, blot dry in the pad of bibulous paper and examine under the microscope.

3.) When using the microscope, do not focus on a specimen by turning the objective lense down toward the slide because the objective lense may jam into the slide and scratch the valuable lense. Instead, begin by turning the objective downward to the lowest position (watch from the side of the microscope) and then focus by turning away from the specimen while you look through the eye piece. Also, it is easier to locate the specimen under a low power objective and then turn to consecutively higher power objectives.

4.) Examine each organism under the microscope and draw pictures of stained microorganisms in a chart (see below). Discard the flat glass microscope slides when you are finished.

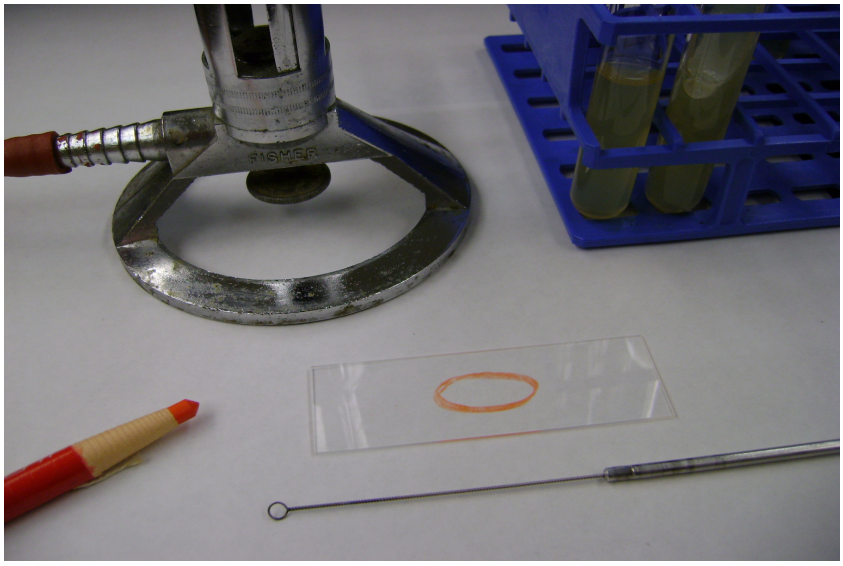

Figure 1. Delivering Culture to a Slide

Results:

TABLE 1. Simple Stain of Biofuel Producing Microorganisms

Organism	450X Power	1000X Power (oil immersion)

Note: Your chart should be larger to allow space for drawings.

Questions and Comments:

What do you think is the purpose of heat-fixing bacteria to a slide for staining? (Heat-fixing sticks the bacteria on the slide so that they will not wash off. Would unstained bacteria be easy to observe with the microscope and why? (Unstained bacteria can be difficult to see under the microscope because they are relatively transparent.) What cell morphology and configurations did you observe for each organism? (*E. coli, K. pneumoniae,* and *C. acetobutylicum* are rods that usually exist as singles or pairs. *S. albus* is a rod that prefers to grow in chains. *S. cerevisiae* shows a large individual egg-shaped cell.)

Further Reading:

Alternative Fuels Curriculum for Pre-College Education.
http://eng-sci.udmercy.edu/pre-college/alt_fuel_curriculum/index.htm.

American Society for Microbiology. www.asm.org (Education, Journals, Public Policy, Searches and Special Interests).

Ingram, L. O., T. Conway, and F. Alterthum. March 1991. Ethanol production by *Escherichia coli* strains co-expressing *Zymomonas* PDC and ADH genes. U.S. patent 5,000,000.

Leboffe, M. J. and B. E. Pierce. 1999, 2005. *A Photographic Atlas for the Microbiology Laboratory*, Morton Publishing Company.

Prescott, L. M., J. P. Harley and D. A. Klein. 2008. *Microbiology*, McGraw-Hill Companies. www.mhhe.com/biosci/cellmicro/prescott.

Difco Laboratories. 1985. Dehydrated culture media and reagents for microbiology, p. 619-620, 622-623, 768-772, 951-956. *In* The Difco Manual, 10th ed. Difco Laboratories, Detroit, Mich.

Kalscheuer, R., T. Stolting, and A. Steinbuchel. 2006. Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology* 152: 2529-2536.

Murarka, A., Y. Dharmadi, S. S. Yazdani, and R. Gonzalez. 2008. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. *Appl. Environ. Microbiol.* 74: 1124-1135.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991. Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. *Appl. Environ. Microbiol.* 57:2810-2815.

Olukoshi, E.R., and N.M. Packter. 1994. Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* 140:931-943.

Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. *J. Bacteriol.* 176:1443-1450.

Trinh, C. T. , and F. Srienc. 2009. Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. *Appl. Environ. Microbiol.* 75:6696-6705.

Sources for Materials:

- Carolina Biological Supply Company
  P. O. Box 6010
  Burlington, NC 27216
  (800) 334-5551
  www.carolina.com

- Fisher Scientific Supply
  1600 West Glenlake Avenue
  Itasca, IL 60143
  (800) 766-7000
  www.fishersci.com

- Presque Isle Cultures
  P.O. Box 8191
  Presque Isle, PA. 16505
  (814) 833-6262
  www.picultures.com

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- Troy Biologicals Incorporated
  1238 Rankin
  Troy, MI 48083
  (800) 521-0445
  www.troybio.com
  (microbiological media)

Experiment #2: HOST BACTERIA FOR BIOFUEL SYNTHESIS, AND BIODIESEL GLYCEROL BYPRODUCT

Purpose: this project examines the ability of bacteria commonly used for genetic engineering to utilize biodiesel glycerol byproduct.

Background: *Escherichia coli* and *Klebsiella* species have become important in biofuel research because strains (containing foreign genes) have been constructed by genetic engineering that produce ethanol (Ingram et al., 1991; Ohta et al., 1991; www.ethanolrfa.org). Moreover, *E. coli* has recently been genetically engineered to produce butanol and biodiesel respectively (Connor and Liao, 2008; Kalscheuer et al., 2006). These bacteria are known to naturally utilize pure glycerol as a carbon source. Consequently, it could be profitable to screen representatives of these species for their ability to utilize biodiesel glycerol byproduct as feedstock.

Items to know:

- Biodiesel glycerol byproduct: three liters of biodiesel are produced for every liter of biodiesel glycerol byproduct (www.biodiesel.org; www.biomich.com.).
- Doubling time or generation time: time to double
- Maximum bacterial concentration: about 3 X 10^9 cells/ml (2^n where n = # generations)
- Differential medium: medium that enables the investigator to distinguish one species from another. This is usually done through use of a chemical reaction which causes the color of the medium or colonies to change.
- Fermentation broth: this is a test medium to determine if an organism can utilize a specific carbohydrate. The broth includes phenol red to test for acid produced from utilization/fermentation of the carbohydrate (Fig. 1). The broth also contains proteanatious material to support growth, but no acid is produced from this material.
- Minimal broth: a medium containing only a few simple chemicals from which a cell must support all the functions of life.
- Colorimeter: a device that measures the turbidity of a culture – a greater number of cells increases the turbidity.
- Colony count assay: samples of diluted culture are spread over surfaces of agar in Petri plates. After incubation, the number of colonies can be used to calculate cell concentration.

Materials:

1 of each culture per pair/table:
Escherichia coli K12 (25°C nutrient agar slant or plate culture)
Cultures can be purchased directly from a biological supply house or grown fresh with standard culture medium.

5 10 ml tubes of phenol red broth base (This medium does not contain added carbohydrate/carbon source)
1 20% stock solution of glucose
1 20% stock solution of sucrose
1 20% stock solution of glycerol
1 20% stock solution of biodiesel glycerol byproduct – pH adjusted to 7.4
(glycerol byproduct can be obtained from a producer of biodiesel or made by use of a "personal batch technique" - www.making-biodiesel-at-home.com/Making_Biodiesel_in_your_kitchen.html)
1 Stock bottle minimal salts broth (Miller,1972)
Substitution of NaCl for citrate by weight will permit the use of the medium in experiments with bacteria that utilize citrate. *E. coli* does not utilize citrate.
1 2% stock solution of $MgSO_4$ - $7H_2O$
1 .05% stock solution of B1 (thiamine hydrochloride)
E. coli K12 requires B1
5 16 x 150 mm culture tubes with caps
5 15 x 123 mm colorimeter tubes with caps
6 10 ml pipets
6 1 ml pipets
1 Propipet
1 Test tube rack
1 Inoculating loop
1 Bunsen burner
1 Roll of label tape
1 Wax pencil/marker
1 Klett-Summerson Colorimeter (blue filter)
A spectrophotometer (wavelength set to 650 nm) can substitute.
1 Sheet of graph paper or Microsoft Office Excel
All glassware and solutions must be must be sterile.

Methods:

Phenol red broth assay

1.) Label one tube of phenol red broth as no carbon source added. Dispense one-tenth ml of sucrose, glycerol, and glycerol byproduct solutions to three additional tubes of phenol red broth respectively and label the tubes accordingly. (If glycerol byproduct is not available, plain glycerol can serve as a substitute.) With a loop, inoculate each tube with a small amount of *E. coli* K12 culture and mix.

2.) Incubate the tubes for 2 days at 25^{o}C (room temperature) or 18 h at 37^{o}C (incubator), and record the results in Table 1 below. If it is wished, an additional, different bacterium may also be screened.

Minimal broth assay

1.) Dispense 10 ml of minimal salts broth to teach of five 16 x 150 mm culture tubes. Subsequently add .1 ml of the $MgSO_4$ and B1 solutions to each of the tubes. Add .1 ml of the glucose, sucrose, glycerol, and biodiesel glycerol byproduct solutions to tubes two through five respectively. Mix all tubes. (Plain glycerol can serve as a substitute if biodiesel glycerol byproduct is not available.)

2.) Dispense 6 ml from each tube above to a respective colorimeter tube and label each tube by the carbon source. Label near the cap so that interference with the colorimeter function will not occur. (Prepare a second set of tubes to be to use as uninoculated zero blanks in the colorimeter.) With a loop, add a small inoculum of *E. coli* K12 culture so that an optical density (OD) reading of about 7 units (about 5.0 x 10^7/ml bacteria) is reached after mixing. It is helpful to suspend the bacteria in the medium by smearing the inoculum against the tube wall near the meniscus.

3.) Incubate the tubes at 25^{o}C and daily record culture OD readings in Table 2 below. Typical readings are provided for a culture that uses glucose as the carbon source. After a week, plot the data by use of Microsoft Office Exel or a piece of graph paper (Fig. 2).

Results:

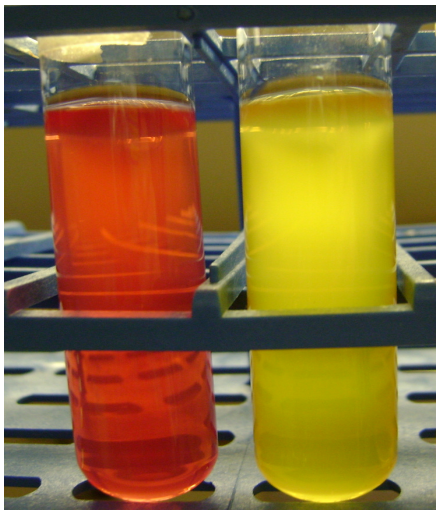

Figure 1. Fermentation Reactions in Phenol Red Broth
Left, nonfermentation of carbon source; right, fermentation of carbon source.
Incubation at 37°C for 18 hrs.

TABLE 1: Fermentation of Glycerol Byproduct in Phenol Red Broth by *E. coli* K12

	Carbon Source				
Microorganism	None	Glucose	Sucrose	Glycerol	Biodiesel byproduct
E. coli K12	(-)	(+)			
Other					

Note: (-), Nonfermentation of carbon source (red); (+), fermentation of carbon source (yellow).
Concentration of added carbon source was .2%.
Incubation at 25°C for 48 hrs.

TABLE 2. Culture Optical Densities for *E. coli* K12 in Minimal Broth containing Biodiesel Glycerol Byproduct

	Carbon Source				
Days incubation	None	Glucose	Sucrose	Glycerol	Biodiesel byproduct
0	8	8			
1	7	13			
2	7	20			
3	8	149			
6	9	117			
7	4	119			

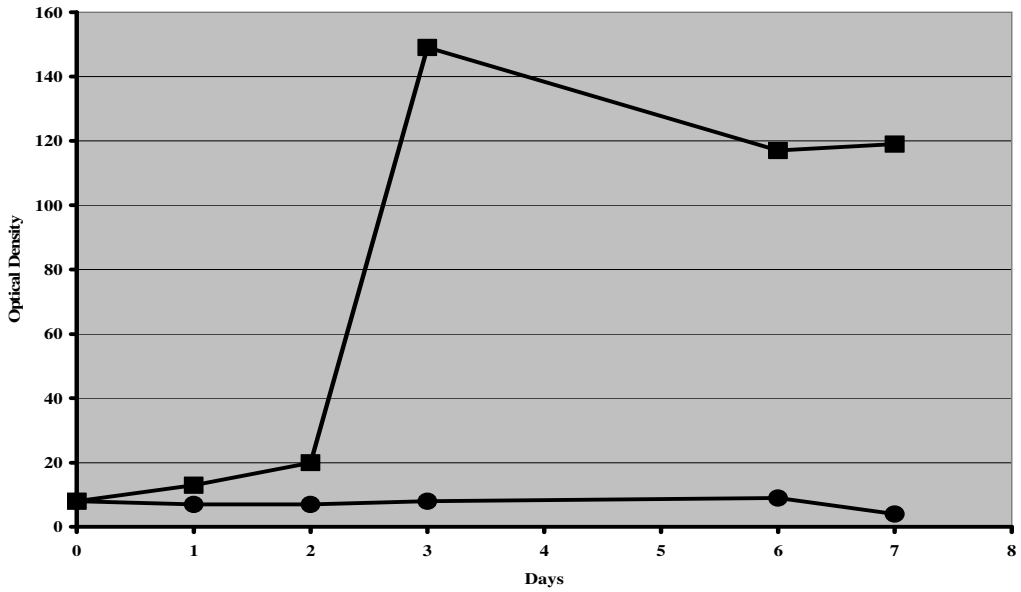

Figure 2. Growth of *E. coli* K12 in Minimal Broth containing Biodiesel Glycerol Byproduct
Circle, no carbon source; square, glucose.
Incubation at 25°C.

Questions and Comments:

What difference is there in carbon source utilization between the phenol red and minimal broth? (In minimal broth the carbon source added is the only carbon source available for growth.) Does a colorimeter and spectrophotometer detect live or dead bacteria? (These instruments measure both live and dead cells.) What is a method to detect cell growth and assess only live cells? (The colony count assay detects only live cells but incubation time is required before data can be retrieved.) Did sucrose, glycerol or biodiesel product serve as a good carbon source in the phenol red or minimal broth? (*E. coli* K12 cannot utilize sucrose but can grow with glycerol or biodiesel byproduct. However, glucose appears to work as the best food source.) It has been reported that *E. coli* bacteria can produce ethanol – an important biofuel (Trinh, and Srienc. 2009).

Further Reading:

Alternative Fuels Curriculum for Pre-College Education. http://eng-sci.udmercy.edu/pre-college/alt_fuel_curriculum/index.htm.

American Society for Microbiology. www.asm.org (Education, Journals, Public Policy, Searches and Special Interests).

Connor, M. R., and J. C. Liao. 2008. Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. *Appl. Environ. Microbiol.* 74:5769-5775.

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Ingram, L. O., T. Conway, and F. Alterthum. March 1991. Ethanol production by *Escherichia coli* strains co-expressing *Zymomonas* PDC and ADH genes. U.S. patent 5,000,000.

Kalscheuer, R., T. Stolting, and A. Steinbuchel. 2006. Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology* 152: 2529-2536.

Leboffe, M. J. and B. E. Pierce. 2006. *Microbiology Laboratory Theory and Application*, 2nd ed, Morton Publishing Company, Englewood, CO.

Miller, J. H., ed. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Murarka, A., Y. Dharmadi, S. S. Yazdani, and R. Gonzalez. 2008. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. *Appl. Environ. Microbiol.* 74: 1124-1135.

Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram. 1991. Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. *Appl. Environ. Microbiol.* 57:2810-2815.

Prescott, L. M., J. P. Harley and D. A. Klein. 2008. *Microbiology*, McGraw-Hill Companies. www.mhhe.com/biosci/cellmicro/prescott.

Trinh, C. T. , and F. Srienc. 2009. Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. *Appl. Environ. Microbiol.* 75:6696-6705.

Sources for materials:

- Carolina Biological Supply Company
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  Burlington, NC 27216
  (800) 334-5551
  www.carolina.com
- Fisher Scientific Supply
  1600 West Glenlake Avenue
  Itasca, IL 60143
  (800) 766-7000
  www.fishersci.com
- Presque Isle Cultures
  P.O. Box 8191
  Presque Isle, PA. 16505
  (814) 833-6262
  www.picultures.com
- Sigma –Aldrich Corporation
  P.O. Box 14508
  St. Louis, MO 63178
  (800)-325-3010
  www.sigma-aldrich.com
- Troy Biologicals Incorporated
  1238 Rankin
  Troy, MI 48083
  (800) 521-0445
  www.troybio.com (microbiological media)

Experiment #3: BUTANOL PRODUCTION, AND BIODIESEL GLYCEROL BYPRODUCT

Purpose: butanol producing bacteria are to be cultured and the effect of biodiesel glycerol byproduct examined.

Background: Butanol is a four carbon alcohol that can be used in production of important products such as plasticizers, lacquers and resins. Butanol currently has value for potential use as fuel extender or fuel (www.butanol.com). Acetone-butanol-ethanol (ABE) microbial fermentation was the main source of butanol and acetone until the early 1950s when it became more cost effective to produce these chemicals from the petroleum industry. However, the supply of petroleum is expected to decrease. As the price of gas from petroleum goes up, butanol may be made by the microbial fermentation industry again. This would be good for farmers because the basic substrate (feedstock) for butanol is starch (grains). Butanol is made by the anaerobic bacterium known as *Clostridium acetobutylicum*. Synthesis of product by the organism has two phases (Jones and Woods, 1986). (1.) In acidogenesis there is vegetative growth where organic acids acetate and butyrate are produced. (2.) In solventogenesis, acids produced are transformed into acetone, butanol and ethanol. Identification of new and inexpensive carbon (nutrient) sources for butanol production could decrease cost.

Items to know:

- Biodiesel glycerol byproduct: three liters of biodiesel are produced for every liter of biodiesel glycerol byproduct (www.biodiesel.org; www.biomich.com.).
- Aerobe: a microorganism that needs oxygen for growth.
- Anaerobe: a microorganism that can thrive in the absence of oxygen.
- Methods for growing anaerobes: anaerobic chamber or glove box, anaerobic jar, roll tube and thioglycolate broth tube.
- Fluid thioglycolate medium (FTM): a medium used for the cultivation of some of the less strict anaerobic bacteria such as clostridia (Difco Laboratories, 1985). Sodium thioglycolate decreases the oxidation reduction potential (Eh) to assist growth of anaerobic bacteria.
- Oxyrase For Broth: a commercial enzyme system used to help create anaerobiosis (the absence of oxygen). Superoxide dismutase is an enzyme to help protect anaerobic bacteria from oxygen toxicity.

Materials:

1 culture per pair:

Clostridium acetobutylicum in fluid thioglycolate medium (FTM)
Cultures can be purchased directly from a biological supply house or grown fresh with standard culture media.

8 10 ml broth tubes of plain FTM with screw caps (plain fluid thioglycolate medium is manufactured without glucose and is used for fermentation studies; Difco Laboratories, 1985)
1 1ml Oxyrase For Broth (Oxyrase, Inc.)
1 20% stock solution of glucose
1 20% stock solution of glycerol
1 20% stock solution of biodiesel glycerol byproduct – pH adjusted to 7.4
(glycerol byproduct can be obtained from a producer of biodiesel or made use of a "personal batch technique" - www.making-biodiesel-at-home.com/Making_Biodiesel_in_your_kitchen.html)
1 Roll pH paper range 4-9 (Hydrion)
8 Glucose test strips (Precision Labs, Inc.)
8 Alcohol test strips (AlcoScreen)
13 1 ml sterile pipets
2 Sterile Pasteur pipets
1 Roll of paper towels
1 Wax pencil/marker
1 Bunsen burner
1 Roll of tape for labeling tubes
All glassware and solutions must be must be sterile.

Methods:

1.) To prepare a set of experimental tubes, unscrew the caps and dispense .2 ml Oyrase For Broth product into each of 4 tubes of plain FTM with a pipet. Gently stir the contents of the first tube with the pipet. Dispense .1 ml of 20% solutions of glucose, glycerol, and biodiesel glycerol byproduct into the last three tubes respectively with three different pipets. (If glycerol byproduct is not available, the glycerol can serve as a substitute in the exercise.) Gently stir the contents of the tubes with the respective pipets as the contents are being released. (Do not stir the broth excessively as this will add extra oxygen from the air!) Screw the caps on snug and wait 30 - 60 minutes for the Oxyrase to remove oxygen.

The inclusion of Oxyrase appears to enhance growth of *C. acetobutylicum*. Prepare a second set of broth tubes to be used as uninoculated controls. Addition of the glycerol byproduct to the broth may cause cloudiness, but it should still be possible to recognize culture growth.

2.) Momentarily, loosen the cap on the stock culture and the 4 experimental tubes of broth to be inoculated. With a new pipet, remove .1 ml of *C. acetobutylicum* stock culture. Insert the pipet to the bottom of the first broth tube and release the inoculum stock culture as the pipet is withdrawn. Repeat the manipulation for the remaining three broth tubes using a new pipet each time.

Note: if it is wished, the tests described below could just be performed on the stock culture purchased from the biological supply company and a tube of uninoculated medium.

3.) Incubate all the tubes at 25^{o}C (room temperature) for 2 – 4 weeks. Inspect periodically for increase in cloudiness – suggesting growth of culture (Fig. 1). *C. acetobutylicum* grows very slow compared to most bacteria. It is not recommended to stir the cultures during incubation as this could increase the level of oxygen and decrease growth.

4.) After incubation, unscrew the caps on the 4 experimental and 4 control tubes. Tear off 8 pieces of pH paper – each measuring about 3 cm in length and lay them in order on a paper towel. Using a new Pasteur pipet each time, dispense 1 – 2 drops of culture from each tube on to the pH paper strips. The color developed can be matched with the corresponding pH value by use of the scale provided by the pH paper manufacturer. Record these and other results in a table like the one below.

5.) Remove 8 glucose test strips and lay them in order on a paper towel. The strips provide a rapid solid phase chemistry enzyme (glucose oxidase and peroxidase) based assay to detect glucose. Using a new Pasteur pipet each time and deliver a small amount of culture to the small filter paper test pad at the end of the strip. If glucose is present in the culture, the pad will turn green. Read the results in about 3 minutes.

6.) Remove 8 alcohol test strips and lay them in order on a paper towel. These strips provide a rapid solid phase chemistry enzyme (alcohol oxidase and peroxidase) based assay to detect alcohol (see reactions below). The manufacturer suggested that the test can score for alcohols having up to 4 carbons and will not react with glycerol. Using a new Pasteur pipet each time deliver a small amount of culture to the small filter paper test pad at the end of the strip. If alcohol is present in the culture, the pad will turn a shade of blue (Fig. 2). Read the results in about 2 minutes.

$$CH_3CH_2OH \text{ --- Alcohol Oxidase ---} \blacktriangleright \; CH_3\overset{H}{C} = 0 + H_2O_2$$

$$H_20_2 + DH_2 \text{ --- Peroxidase ---} \blacktriangleright \; D \text{ (Blue)} + 2H_20$$

Alcohol Detection Reactions

Results:

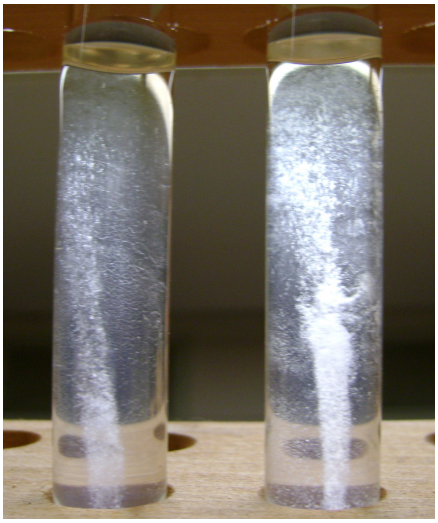

Figure 1. ***C. acetobutylicum*** **Cultured in Fluid Thioglycolate Medium**
Left, no added carbon source; right, glucose added.
Base medium was plain fluid thioglycolate medium without glucose.
Oxyrase reagent was added to both tubes.
Contentration of added carbon source was .2%.
Inoculum was grown in fluid thioglycolate medium and diluted 1/100.
Incubation at 25°C for 4 weeks.
When samples of culture are inoculated on agar plate media and incubated in the presence of air (oxygen), no colonies will grow!

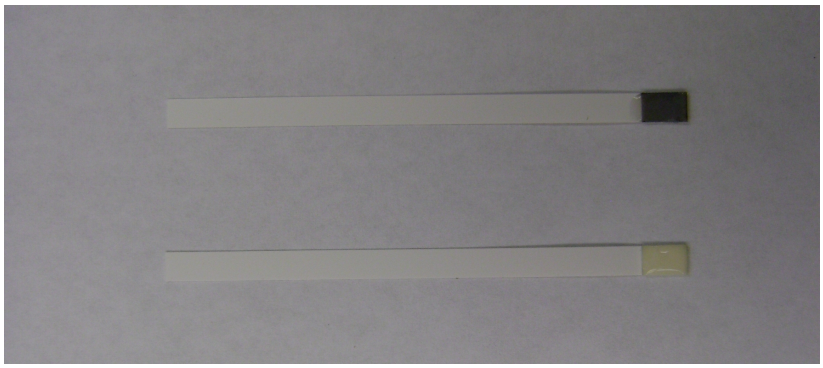

Figure 2. Alcohol Test Strips and Results
Top, strong positive reaction.
Bottom, negative reaction.

TABLE 1. Alcohol production and *C. acetobutylicum* cultures

	pH range		Glucose (+ or -)		Alcohol (+ or -)	
Carbon source	Control	Experimental	Control	Experimental	Control	Experimental
None Added						
Glucose						
Glycerol						
Byproduct						

Note: Control, uninoculated medium.
Experimental, culture in medium.
The pH range, 4 – 9.
(+), Test substate present.
(-), Test substrate absent.
Incubation at 25^{o}C for 2 – 4 weeks.

Questions and Comments:

Which tube appeared to produce the most growth? (The tube containing added glucose appeared to produce the most growth). What is the significance of a low pH in a culture of *C. acetobutylicum*. (In the acidogenesis phase there is vegetative growth where organic acids acetate and butyrate are produced.) How did the experimental glucose tube test for presence of glucose? Explain the result. (The bacteria consumed the glucose and produced alcohol.) Did bacteria grow in the presence of glycerol or glycerol byproduct? (Yes - however, presence of glycerol or byproduct in medium did not appear to increase growth of *C. acetobutylicum*.) The significance of cultures containing glycerol byproduct that test positive for alcohol content is unclear because glycerol byproduct showed a positive test for the presence of alcohol. This project was inspired by the report that production of butanol by *C. acetobutylicum* from glucose was increased in the presence of glycerol (Vasconcelos et al., 1994). The high concentrations of glycerol used in the medium by those investigators may not have provided a carbon source but have increased the medium viscosity to create a desirable environment for growth and production of butanol. Some microorganisms will not grow well in a watery medium. The FTM contains a small amount of agar as thickener. In nature, clostridia are normally found in viscous sediments and mud.

Further Reading:

Alternative Fuels Curriculum for Pre-College Education. http://eng-sci.udmercy.edu/pre-college/alt_fuel_curriculum/index.htm.

American Society for Microbiology. www.asm.org (Education, Journals, Public Policy, Searches and Special Interests).

Difco Laboratories. 1985. Dehydrated culture media and reagents for microbiology, p. 951-956. *In* The Difco Manual, 10th ed. Difco Laboratories, Detroit, Mich.

Girbal, L., and P. Soucaille. 1994. Regulation of *Clostridium acetobutylicum* metabolism as revealed by mixed–substrate steady-state continuous cultures: role of NADH/NAD ratio and ATP pool. *J. Bacteriol*. 176:6433-6438.

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Murarka, A., Y. Dharmadi, S. S. Yazdani, and R. Gonzalez. 2008. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. *Appl. Environ. Microbiol.* 74: 1124-1135.

Prescott, L. M., J. P. Harley and D. A. Klein. 2008. *Microbiology*, McGraw-Hill Companies. www.mhhe.com/biosci/cellmicro/prescott.

Vasconcelos, I., L. Girbal, and P. Soucaille. 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. *J. Bacteriol*. 176:1443-1450.

Woods, D. R. 1995. The genetic engineering of microbial solvent production. *Trends Biotechnol*. 13:259-264.

Sources for materials:

- Carolina Biological Supply Company
  P. O. Box 6010
  Burlington, NC 27216
  (800) 334-5551
  www.carolina.com
  (Precision Labs, Inc. glucose test strips)

- Fisher Scientific Supply
  1600 West Glenlake Avenue
  Itasca, IL 60143
  (800) 766-7000
  www.fishersci.com

- Oxyrase, Inc.
  P.O. Box 1345
  Mansfield, OH 44901
  (419) 589-8800
  www.oxyrase.com

- Presque Isle Cultures
  P. O. Box 8191
  Presque Isle, PA. 16505
  (814) 833-6262
  www.picultures.com

- Sigma –Aldrich Corporation
  P.O. Box 14508
  St. Louis, MO 63178
  (800)-325-3010
  www.sigma-aldrich.com

- Test Medical Symptoms
  6633 Ashman Road
  Maria Stein, OH 45860
  (888) 595-3136
  www.testsymptomsathome.com
  (AlcoScreen Alcohol Test Strips)

- Troy Biologicals Incorporated
  1238 Rankin
  Troy, MI 48083
  (800) 521-0445
  www.troybio.com
  (microbiological media)

Experiment #4: PRODUCTION OF ANTIBIOTIC BY USE OF BIODIESEL GLYCEROL BYPRODUCT

Purpose: making of antibiotic will be performed by incorporating biodiesel glycerol byproduct in the growth media.

Background: Some microbes such as *Streptomyces* bacteria and its relatives naturally contain high levels of triacylglycerols (Olukoshi and Packter, 1994). These microbes may be a prospective source of fatty acids for making biodiesel. However, a process to make biodiesel from these microbes has not been developed. *Streptomyces griseus* is known for production of the antibiotic called streptomycin. It may be possible that *S. griseus* which is a natural utilizer of glycerol as a nutrient, may be grown with biodiesel glycerol byproduct to make lipids for biodiesel, antibiotic or both.

Items to know:

- Biodiesel glycerol byproduct: three liters of biodiesel are produced for every liter of biodiesel glycerol byproduct (www.biodiesel.org; www.biomich.com.).
- Antibiotic: a chemical synthesized by a microbe that will kill or inhibit other microbes.
- Important producers of antibiotics: *Streptomyces*, *Bacillus*, *Penicillium* and *Cephalosporium*.
- Actinomycete isolation agar: a standard complex medium that includes glycerol as a regular ingredient and is used to grow *Streptomyces* species.
- Minimal agar: a medium containing only a few simple chemicals from which a cell must support all the functions of life.
- Brain heart infusion agar: a nutrient rich medium used for the cultivation of a variety of microorganisms.

Materials:

1 of each culture per pair/table:

Streptomyces griseus (25°C nutrient agar slant – will not grow at 37°C)
Escherichia coli K12 (37°C nutrient broth culture – needed two weeks later)
Cultures can be purchased directly from a biological supply house or grown fresh with standard culture media.

1 20% stock solution of biodiesel glycerol byproduct – pH adjusted to 7.4
(glycerol byproduct can be obtained from a producer of biodiesel or made use of a "personal batch technique" - www.making-biodiesel-at-home.com/Making_Biodiesel_in_your_kitchen.html)
1 20% stock solution of glycerol (Glycerol can be used as a substitute if biodiesel glycerol byproduct is not available.)
1 2% stock solution of $MgSO_4$ - $7H_2O$
1 20 ml minimal salts agar plate with NaCl substituted for citrate by weight (Miller, 1972)
1 20 ml actinomycete isolation agar plate – prepared without glycerol (Difco Laboratories, 1985)
1 Bent rod bacteriological sample spreader
1 200 ml beaker or wide-mouth jar containing 50 ml ethanol
3 Sterile swabs
2 10 ml sterile pipets
1 .9% stock solution of NaCl
2 Disposable 5 ml syringes
2 Disposable sterile 25 mm filter sterilization devices with .45 um membrane
2 Sterile 16 mm x 150 mm test tubes with caps
2 1 ml sterile pipets
2 20 ml brain heart infusion (BHI) agar plates for sterility test (Difco Laboratories, 1985)
3 Pasteur pipets
1 10 ml BHI agar plate (surface must be dry)
1 Propipet
1 Wax pencil/marker
1 Bunsen burner
All glassware and solutions must be must be sterile.
See methods below on time to have materials available.

Methods:

1.) Deliver .5 ml of biodiesel glycerol byproduct or glycerol to the surface of a minimal salts agar plate and an actinomycete agar plate. Deliver .5 ml of the $MgSO_4$ solution to the surface of the minimal salts agar plate. Dip the bent rod in the alcohol, touch the rod to the flame and let the alcohol burn off as a means of sterilization. Spread the liquid supplement(s) thoroughly over the surface of each agar plate. Flame sterilize the bent rod between each plate. Place the plates in a 37°C incubator, crack the lids and leave several minutes until the agar surfaces are dry.

2.) To collect inoculum, dab a sterile swab into the slant culture of *S. griseus*. Swab the bacteria back and forth over the entire surface of the minimal salts agar plate. With a new swab repeat the process with the actinomycete agar plate. Be sure to label the backs of the plates. Incubate plates in the inverted position for two weeks at 25°C (room temperature).

3.) Gently deliver 10 ml saline to the surface of each plate culture. The first few drops of saline may form beads on the surface of the microbial mat (Fig.). The culture mat appears to be hydrophobic. It is tempting to suggest that this is associated with the high lipid content of cell. Very slowly swirl the saline in the plate to completely cover the culture mat. Incubate plates in the refrigerator at 4°C for two days to elute any antibiotic.

4.) Unwrap a filter sterilization disc and carefully remove it by only touching the circular edge. (Do not touch the male exit lock of the disc! This must remain sterile.) Remove plate lids and tilt one plate to pool the saline (eluent) on one side. Fill the syringe with 5 ml of the eluent from the plate. Plug the syringe full of liquid into the female lock of the disc. Remove the cap on an empty test tube and hold the male exit lock of the filter sterilization disc - syringe assembly over the open test tube. Gently eject the liquid from the syringe through the disc and down into the test tube. Replace the cap on the tube and label it "filtered". Repeat the process with the second agar plate culture. Label the tubes and store the eluent preparations at 4°C.

5.) Deliver .2 ml of each eluent preparation to the surfaces of two respective BHI agar plates. Spread the samples over the surfaces of the agar plates with a sterile bent rod as described above. Label the back of the plates with contents and incubate at 25°C for two days to test for sterility. If no colonies grow, the preparations of eluent are considered sterile. Record the presence or absence of bacterial colonies in a table like the one below.

6.) Dip a sterile swab into the *E. coli* culture and drain off excess liquid against the side of the tube. Swab the entire surface of the 10 ml BHI agar plate. The thin plate should enhance detection of antibiotic activity. On the back divide the plate into thirds with a marker. Once the surface of the plate has dried, with a Pasteur pipet, add a drop of saline and each eluent preparation to the sections respectively. After the drops have dried, incubate the plate at 37°C for two days. Be sure to have labeled the back of the plate with contents and date. Record the presence or absence of bacterial inhibition in the table.

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Results:

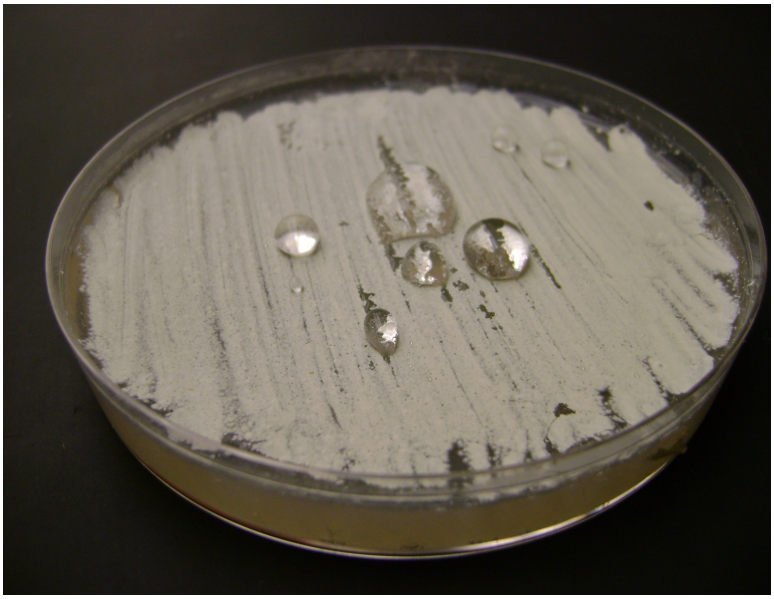

Figure 1. Drops of Normal Saline on Hydrophobic Culture Mat of *S. griseus*
Inculation was performed with a swab.
Actinomycete agar contained .5% byproduct.
Incubation at 25°C for 2 weeks.
Plate was flooded with 10ml saline and stored at 4°C for 48 h before collection of eluent.

TABLE 1. Production of Antibiotic by use of Biodiesel Glycerol Byproduct

	Saline	Minimal agar	Actinomycete agar
Inhibition zone on *E. coli*[a]			
Colonies from eluent (sterility)[b]			

Note: [a], (+), Development of growth inhibition zone; -, no inhibition zone.
[b], (+), Colonies produced; -, no colonies produced.

Draw a sketch or take a photograph of the test plate!

Questions and Comments:

What is the significance of a hydrophobic and hydrophilic substance? (A hydrophobic substance does not mix with water while a hydrophilic substance mixes with water.) The first few drops of saline formed beads on the surface of the microbial mat, instead of spreading out over the surface. Can you provide a possible explanation for this? (The culture mat appeared to be water fearing or hydrophobic. It is tempting to suggest that this is associated with the high lipid content of cell.) Was there any evidence of antibiotic production? (The circular "no growth zone" was an indication that antimicrobial product(s) were produced.) Did the eluent from the minimal agar and the actinomcete agar demonstrate the same antimicrobial ability, and why? (The eluent from the actinomycete agar showed more inhibition, probably because the medium contained more growth factors for the antibiotic producing *S. griseus*.) Name some mechanisms/points of antibiotic action and give examples. (Cell wall agents, penicillin; cell membrane agents, polymyxins; protein synthesis inhibitors, streptomycin; nucleic acid synthesis, rifampicin.)

Further Reading:

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American Society for Microbiology. www.asm.org (Education, Journals, Public Policy, Searches and Special Interests).

Difco Laboratories. 1985. Dehydrated culture media and reagents for microbiology, p. 66-67, 163-164, 619-620. *In* The Difco Manual, 10th ed. Difco Laboratories, Detroit, Mich.

Leboffe, M. J. and B. E. Pierce. 1999, 2005. *A Photographic Atlas for the Microbiology Laboratory*, Morton Publishing Company.

Kalscheuer, R., T. Stolting, and A. Steinbuchel. 2006. Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology* 152: 2529-2536.

Miller, J. H., ed. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Murarka, A., Y. Dharmadi, S. S. Yazdani, and R. Gonzalez. 2008. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. *Appl. Environ. Microbiol.* 74: 1124-1135.

Olukoshi, E.R., and N.M. Packter. 1994. Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* 140:931-943.

Prescott, L. M., J. P. Harley and D. A. Klein. 2008. *Microbiology*, McGraw-Hill Companies. www.mhhe.com/biosci/cellmicro/prescott.

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  (800) 766-7000
  www.fishersci.com

- Presque Isle Cultures
  P. O. Box 8191
  Presque Isle, PA. 16505
  (814) 833-6262
  www.picultures.com

- Troy Biologicals Incorporated
  1238 Rankin
  Troy, MI 48083
  (800) 521-0445
  www.troybio.com
  (microbiological media)

- Sigma –Aldrich Corporation
  P.O. Box 14508
  St. Louis, MO 63178
  (800)-325-3010
  www.sigma-aldrich.com