MICROBIAL CONVERSION OF BIODIESEL BY-PRODUCTS TO BIOFUEL

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2010

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ACKNOWLEDGEMENTS

I am extremely grateful to my advisor Dr. Matthew Fields. Although I had very little background in microbiology, he took me in as one of his graduate students and showed me nothing but continuous support. Thank you for all of your guidance. I truly appreciate the many hours you have given to help me out (especially the all nighters). Thank you for continuing to encourage me as I move toward a new career path in teaching. I would also like to express my gratitude to Dr. Mike Franklin and Dr. Robin Gerlach the other two members of my committee. Thank you, not only for sharing your expertise and advice, but for being so understanding and flexible with my new endeavors. I sincerely appreciate all your support.

Thanks to all my past and present lab mates, Andy, Anitha, Brad, Chiachi, Erin, Elliot, Kara, Kristen, Jake, Logan, Phil, and Melinda, for all your help inside and outside of lab. I love all of you and really appreciate all the support and laughs. I can't wait to have you come present to my high school students. What great role models you all are! I also want to thank everyone in the Microbiology Department and my coworkers at the CBE for their willingness to share ideas and for their constant helpful attitude. I can't imagine a friendlier work environment. Thanks to John Neuman and Ann Willis for all the technical support. I am extremely grateful for all the advice, guidance, and emotional support that Stephanie Cunningham has shown me since day one of grad school. The Molecular Bioscience Program has been a wonderful experience and I any very thankful for the financial support. Finally, I want to thank my family and friends for their love, guidance, humor, and all their emotional support. Thank you, Chad, for all the dinners!

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ABSTRACT

Biodiesel is an alternative liquid transportation fuel derived from biological oils. It is a renewable form of transportation fuel that can be easily integrated into society's current infrastructure. Biodiesel is cleaner burning than petroleum, emitting less carbon pollution and harmful toxins (i.e. sulfur, benzene). One of the major by-products from biodiesel production is crude glycerin. With the increased production of biodiesel, glycerin production will continue to increase. Glycerin was once considered a valuable co-product but now is considered a low-value by-product. In the following study, different co/tricultures of sulfate reducing bacteria (SRB) and methanogens were grown with crude glycerin as a means to convert the waste product into a renewable energy source, methane. The SRBs, *Desulfovibrio vietnamensis* and *Desulfovibrio alcoholovorans 6133,* were grown syntrophically, in different co/triculture combinations, with *Methanococcus maripaludis, Methanoculleus marisnigri,* and *Methanosarcina acetivorans.* Co/tricultures were investigated for the ability to produce methane via the utilization of pure glycerol, fractionated glycerin, and crude glycerin as carbon and energy sources. In order to gain insight into cellular physiology, glycerol, acetate, free fatty acid, and methane concentrations were measured throughout growth. The co/tricultures grew fastest on pure glycerol and experienced a lag phase in growth on fractionated glycerin and longer lag phases when transferred to crude glycerin. However, methane yields were similar on all three carbon sources. Methane production depended on the carbon source and culture composition. Co/tricultures growing on pure glycerol and fractionated glycerin displayed a decrease of methane production as growth rate increased. The opposite was seen with growth on crude glycerin. With most cultures, the addition of *M. acetivorans* increased methane concentrations significantly. *M. acetivorans* displayed the capability of utilizing the by-product, acetate, from SRB oxidation of glycerol and the methanol layer from fractionated and crude glycerin. *M. acetivorans* appeared to interfere with the coculturing of *D. vietnamensis* and *M. marisnigri* based on decreased methane production. Cocultures with *M. maripaludis* grew poorly and produced little methane when grown on the supernatant of *M. acetivorans.* This is the first study to characterize the utilization of crude glycerin from biodiesel production by syntrophic cultures of SRB and methanogenic archaea.

INTRODUCTION

Transportation Energy.

Hydrocarbon Fuels.

Of the three types of naturally occurring hydrocarbon fuels, carbon is the main element (based on weight) composing all of them: petroleum is 82-87% carbon, natural gas is 75% carbon, and coal is 65-95% carbon (Schobert, 1990). It is widely accepted by scientists that hydrocarbon fuel sources were generated by once living organisms, and this is supported by the presence of certain biomarkers (*e.g.*, chlorophyll and heme) that are often associated with petroleum and coal. Optically active compounds are also observed in petroleum; molecules that are only produced by living organisms (Schorbet, 1990; Thom, 1929). One of the more obvious signs of organic life is the presence of plant remains that are usually found within coal (Schobert, 1990). Autotrophy is the process that incorporates carbon into the biological systems; therefore, it is also responsible for producing the precursors for hydrocarbon fuel. Photosynthesis (equation 1.1) is a process that fixes CO_2 into organic life. The following equation shows the reaction of photosynthesis (Tissot & Welte, 1978):

$$6CO_2 + 6H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (1)$$

Essentially, photosynthesis converts solar energy into chemical energy. This is a non-spontaneous reaction that requires a great amount of energy to proceed; ΔG is equal to

2,720 kJ at 298°K (Schorber, 1990). Approximately 470 kJ is absorbed for every gram mole of carbon that gets fixed into biomass (Klass, 1998). The energy that goes into making glucose ($C_6H_{12}O_6$), a major building block of organic matter, gets stored as chemical energy. Glucose is a metabolic precursor for many cellular components such as amino acids, nucleotides, fatty acids, carbohydrates, etc. As glucose enters one of these physiological pathways, its stored chemical energy gets transferred into other molecules that contribute to the composition of organic matter. Since organic matter has such a great amount of stored energy, and if it does not decay completely, it has the potential to become a hydrocarbon fuel source. The trapped energy can then be harnessed through combustion (the breaking of chemical bonds) and utilized for transportation, heating, electricity, etc. (Schobert, 1990).

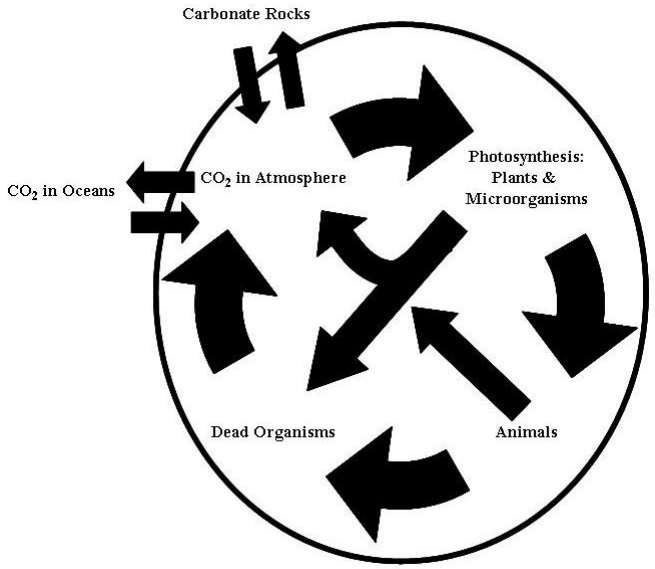


Figure 1. Global Carbon Cycle. Carbon in the atmosphere is in equilibrium with the oceans and within rocks. Carbon in the atmosphere can also be photosynthesized by plants and microorganisms which can then go on into animals. Eventually the organisms will die releasing carbon back into the atmosphere completing the cycle (Schobert, 1990).

The flow of carbon throughout our environment can be summed up in the carbon cycle. Figure 1, a very simplified version of the global carbon cycle, shows how atmospheric carbon (CO_2) gets fixed into biological material through photosynthesizing plants and microorganisms. Through consumption, carbon is transferred throughout the biological systems. When plants, microorganisms, and animals die, they decay and release carbon back into the atmosphere. In some cases, the dead organisms will not fully decompose, and as a result, will eventually become fossil fuels. These fossil fuels are what we use today as hydrocarbon fuels because they still contain the energy trapped from photosynthesis. In order to account for all carbon on Earth, Figure 1 also shows the equilibrium of carbon in the ocean (CO_2) and rocks (carbonate) with the atmospheric carbon. However, this aspect of the carbon cycle is not relevant to hydrocarbon fuel production (Schobert, 1990).

Photosynthesis is responsible for the production of large amounts of organic matter. Although almost 95% of all organic carbon (from dead organisms) returns to the atmosphere, approximately 5% will get buried and become organic sediment. As buried organic sediment, the carbon will either get converted into petroleum or the sediment will erode and the carbon will get oxidized back to CO_2. In order for organic matter to become hydrocarbon fuel it needs to go through at least one of the following processes: diagenesis, catagenesis, and metagenesis (Figure 2) (Schorber, 1990; Tissot & Welte, 1978).

As organic sediments are slowly buried they become more protected from oxidation. The first process of diagenesis is rotting which occurs around 0 to 1 meter

below the surface. In this zone, oxygen is still present so it is basically a competition between the burial and oxidation of organic matter. The organic sediment that makes it past this zone then enters the moldering zone which is located 1 to 2 meters below the surface. The oxygen concentration is much lower at this level so oxidation of the organic sediment does not usually occur. Instead, environmental hydrolysis of larger polymers (i.e. cellulose, proteins) dominates. Through this process, molecules such as carbohydrates, proteins, and glycosides get converted to humus, while lignin, lipids, and resins remain conserved. Putrefaction occurs in the next zone which is 2 to 10 meters below the surface. This is an anaerobic environment where microorganisms will begin to degrade organic material that has entered this zone. Methane, carbon dioxide, and water are common byproducts at this stage. Eventually, when putrefaction is over, the organic sediment becomes an "organic soup" (Schorber, 1990, p. 33) of humic acids. The final left over organic matter, or "organic soup", gets polymerized into kerogen, a non-soluble, high molecular weight substance. The production of kerogen is the final stage of diagenesis, which occurs between 100 to 200 meters below the surface. The generation of kerogen marks the start of transitioning organic material into hydrocarbon fuels (Schobert, 1990; Tissot & Welte, 1978). It is important to note that there is also a path in diagenesis that results in geochemical fossils. In this case, organic material is not altered; either carbon skeletons are retained or organic material is completely unaltered (i.e. lipids) (Tissot & Welte, 1978).

As kerogen gets buried deeper and deeper, it enters the catagenesis phase. This process occurs within the Earth at high temperatures and pressure. Kerogen begins to

degrade into low to medium molecular weight hydrocarbons as the temperature and pressure begins to increase. These smaller hydrocarbon molecules are linear, cyclic, and branched (oil formation stage). As the depth increases so does the temperature, and when kerogen gets to a certain depth some of the carbon-carbon bonds begin to crack. This produces very low molecular weight products like methane and hydrogen sulfide (wet gas formation stage). There are two types of hydrocarbons created during this process: high hydrogen and low hydrogen molecules. The higher hydrogen forming molecules are crude oil and methane which have H:C atomic ratios of 1.5 to 2 and 4, respectively. The left over kerogen is losing hydrogen as the other molecules are produced, and therefore has a H:C ratio of 0.5. When kerogen gets buried even deeper, it enters metagenesis. During this phase, kerogen starts to become restructured to a higher ordering material and only methane is produced (dry gas formation) (Tissot & Welte, 1978; Schobert, 1990; Sokolov, 1972). Figure 2 summarizes the process of diagenesis, catagenesis, and metagenesis; displaying the relative depth and products generated (Rondeel, 2002).

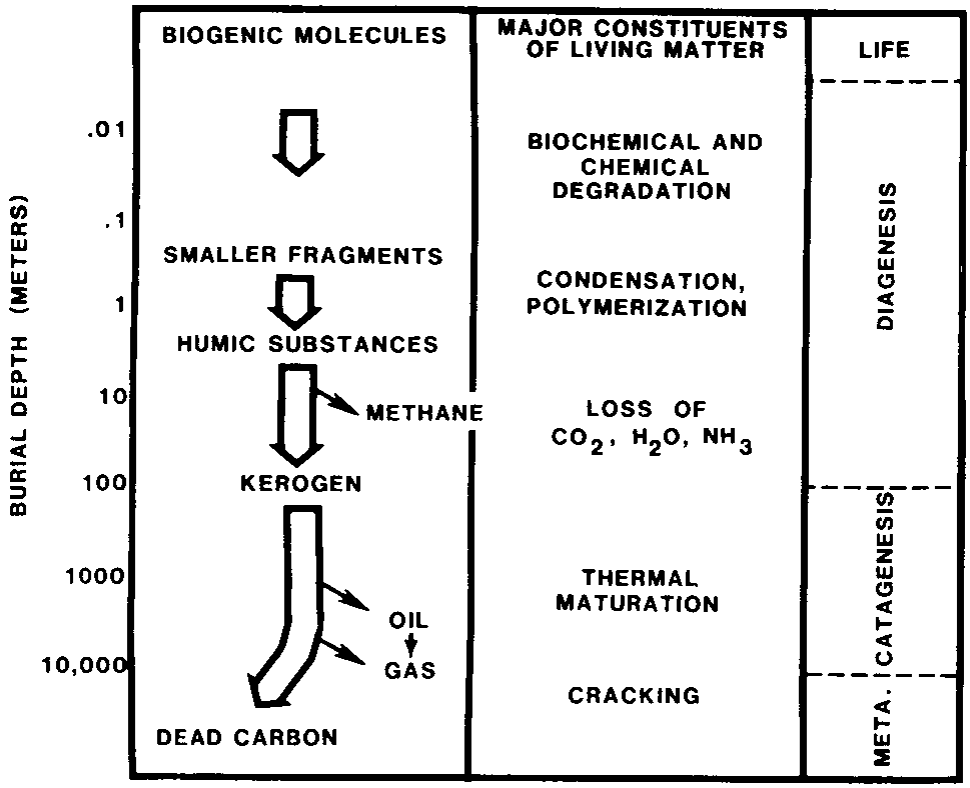


Figure 2. Process of Hydrocarbon Production from Organic Matter. As organic sediments are buried the temperature and pressure increase producing hydrocarbons. Diagenesis, catagenesis, and metagenesis are the different phases that organic sediments go through to become the various molecules of hydrocarbons (Rondeel, 2002).

Petroleum. The following experiment focuses on transportation energy, a sector that utilizes the hydrocarbon fuel, petroleum. Petroleum, or crude oil, can be used for either harnessing energy through burning, like with transportation, or it can be used to produce different materials, like plastics and rubber (Pauling, 1970). Petroleum is a darkish black, viscous liquid made up of many different hydrocarbons. The mixture of hydrocarbons is made up of four different types of components, based on organic solubility: saturates (i.e. alkanes, cycloalkanes), aromatics, resins (aggregates with pyridines, quinolines, carbazol, etc.) and asphaltenes (aggregates with polyaromatics,

naphthenic acides, sulfides, etc.) (Pauling, 1970; Sugiura, Ishihara, Shimachi, & Harayama, 1997; Tissot & Welte, 1978). Since petroleum is composed of hundreds to thousands of compounds, it is necessary to separate them into useful fractions. The process of separating petroleum into different products occurs at an oil refinery (Schobert, 1990). Distillation is a common method for crude oil fractionation which separates compounds based on boiling points.

Gasoline (C_5-C_9 alkanes) generally has the lowest boiling point so it is the first product distilled. On average, petroleum is usually only composed of 10 to 30% gasoline. This is not a high enough percentage to meet the current demand of gasoline. There are two processes that can increase the percentage of gasoline collected from petroleum: thermal/catalytic cracking and molecular building. Thermal and catalytic cracking involve breaking molecules that are greater than C_9 into the carbon range of gasoline. This method can increase the gasoline yield to 40 or 50%. The other process uses polymerization or alkylation to combine smaller carbon molecules within the crude oil to form C_5-C_9 alkanes. Another common transportation fuel is diesel. Diesel fuel comes from the kerosene and gas oil fractions of petroleum. This is a lower quality fuel that ranges from C_{15} to C_{17} alkanes; mostly paraffin and naphthene hydrocarbons. Diesel has a higher density and boiling point than gasoline. Due to its longer saturated chemical composition, it will sometimes solidify when stored. (Schobert, 1990; Babich & Moulijn, 2002; Sokolov, 1972).

Numerous problems associated with the use of petroleum exist. For one, it is predicted that the rate of consumption will eventually exceed the world's supply, and

consequently, it will become more expensive. According to Pahl, the global production of oil will never be able to exceed 100 million barrels a day; however, by 2025 the demand is projected to be around 121 million barrels a day. Currently, the world's largest oil fields are exhausting at a rate of 5% each year (Pahl, 2008). The fact is, there exists only a finite amount of oil reservoirs on our Earth, all of which are guaranteed to run out. In terms of the environment, petroleum is not environmentally friendly. In 1997, fossil fuels were burned at a rate that released 4.4×10^{19} g of carbon into the atmosphere. This is about 400 times more carbon than primary producers are capable of fixing (Dukes, 2003; Pahl, 2008). Aside from CO_2 pollution, petroleum also releases toxic chemicals into the atmosphere such as aromatic compounds, sulfur, nitrogen oxides, and heavy metals. Oil spills are another huge problem that cause substantial amounts of environmental damage; ruining whole ecosystems, killing animals, polluting water, etc. (Sugiura et al., 1997). A case in point is the 2010 British Petroleum spill in the Gulf of Mexico. As petroleum continues to create social and environmental issues the prospect of finding alternative fuels has become more urgent.

Biodiesel.

Rudolf Diesel is the inventor of the diesel engine. His unique idea of the "compression engine" was patented on February 28, 1892 and was based upon ignited fuel through the compression in the presence of air. Diesel built his first prototype in 1893 but it was short lived when the pressure gauge exploded. It was not until 1896 that Diesel designed a working engine. Manufacturers immediately heard and were excited about the new invention. Diesel ended up selling the manufacturing license to a rich man

from the U.S., named Adolphus Busch, for what would be the equivalence of five million dollars today. Using Diesel's patent, Busch built the first commercial diesel engine. During a Paris Exposition in 1900, the engine was shown to run on a variety of fuels including peanut oil. Although petroleum was beginning to dominate the fuel industry by 1911, Diesel was a large supporter for vegetable based fuels and always intended his engine to run off of vegetable oil. Diesel also dreamed of selling his engines to smaller businesses and he believed it could even transform the railway system. Sadly, due to the large engine size, Diesel had a lot of trouble marketing his engine to these smaller businesses. By 1912, Diesel had sold more than 70 thousand engines, but mostly to large businesses. One goal he was able to accomplish was that his engine did go on to revolutionize the railway system after World War II. Unfortunately, Diesel never lived to see this. In fact, his death is very mysterious. In 1913, Diesel was on a ship heading to England for a meeting with the British Diesel Company when disappeared mid-sail in the middle of the night. No one knows what happened to him, but some believe he was extremely depressed about financial problems and decided to end his life. He also had heart problems, so some believe he died of a heart attack and then fell overboard. Others wonder if perhaps, there was a political motive, and that the petroleum industry was somehow involved with his sudden and unexpected disappearance (Pahl, 2008).

In essence, the invention of biodiesel coincided with the invention of the diesel engine. However, by the early 1900's, petroleum, a cheap accessible alternative, begun to take over as the main energy source replacing natural oils. Over time, diesel engines became more compatible with petroleum so that natural oil-fuels became too viscous for

utilization. Today, biodiesel is making a comeback. In order to overcome the high viscosity problem of natural oils so they can be used in modern diesel engines, they must go through a process called transesterification (see below) (Sheehan et al., 1998). A variety of natural oils can be used in biodiesel production some of which come from: palm, coconut, jatropha, rapeseed/canola, camelina, peanut, sunflower, safflower, mustard, soybean, hemp, corn, used cooking oil, animal fat, and algae (Pahl, 2008). Biodiesel is an environmentally friendly alternative to petroleum-based fuel that releases 41% less greenhouse gas emissions (Hill et al., 2006).

When crude oil reaches the price of $45/barrel, biodiesel becomes economically feasible based upon current economic indicators (McGraw, 2009). In July 2008, prices skyrocketed reaching $147/barrel, and as of July 3, 2010, the cost of crude oil was $72.3/barrel (Oil-price.net). The infrastructure for storage and mass distribution of biodiesel already exists, and it can be used in modern engines without any modifications. Many countries have already implemented this alternative fuel into their transportation system. In 2006, Europe generated 85% of the world's biodiesel (1.7 billon gallons produced worldwide). The EU has over 185 biodiesel plants that are capable of producing about 3.3 billion gallons per year. After Germany, the U.S. produces the second highest amount of biodiesel. The National Renewable Energy Lab (NREL) believes the U.S. has the potential to produce 1 billion gallons of biodiesel each year (Pahl, 2008). The current widespread use of biodiesel provides strong evidence for its capability as an alternative to transportation fuel. Rudolf Diesel understood the potential of biodiesel in 1912 when he

wrote, “The use of vegetable oils may become in the course of time as important as petroleum and coal tar products of the present time.” (Klass, 1998, p. 335; Phal, 2008).

Current Issues with Biodiesel and its By-product Crude Glycerin.

Alternative Transportation Energy.

Petroleum has been utilized as the main source of transportation energy in the U.S. since the industrial revolution. In 2008, 28% of the U.S.’s total energy was consumed by the transportation sector which exhausted 9 million barrels of gasoline per day (U.S. Energy Information Administration, 2009). As our consumption of petroleum has increased, we have become more petroleum-dependent, and therefore face issues involving supply shortages, foreign governments, high costs, and environmental pollutant emissions (López, Santos, & Pérez, 2009). In response to these concerns, the Energy Independence and Security Act of 2007 was instated in order to significantly increase the production of alternative fuel by 2022 to 36 billion gallons (Peralta-Yahya & Keasling, 2010). Biodiesel, derived from biological sources, is a promising renewable form of fuel that can be easily integrated into society’s current energy infrastructure; engine, transportation, and storage compatibility. Biodiesel emits less carbon pollution due to its more complete combustion and contains less contaminates (i.e. sulfur, aromatic compounds, metals, etc.) than petroleum based fuels and thus burns cleaner (Peralta-Yahya & Keasling, 2010; Vasudevan & Briggs, 2008). As the top biodiesel producer in the world, the European Union generated approximately 3.2 million tons in 2005. In 2008, Brazil passed a law that made it mandatory for all diesel fuel to be mixed with 2%

biodiesel, and by 2013 the mixture ratio will be required to move up to 5% (Silva, Mack, & Contiero, 2009). Worldwide use of biodiesel reveals its capability of replacing petroleum as a major source of alternative transportation fuel.

Crude Glycerin. Biodiesel can be produced from oil crops (i.e. soybean, palm, rapeseed), cooking wastes (i.e. animal fats, grease), and more recently, microalgae (Vasudevan & Briggs, 2008). All these sources store energy in the form of triacylglycerols; three long chain fatty acids bound to a glycerol backbone via ester bonds (Figure 1.3) (Silva et al., 2009). Triacylglycerols are too viscous for today's car engines that have been modified to run on petroleum, a much less viscous fuel (Sheehan, Dunahay, Benemann, & Roessler, 1998). In order to overcome the high viscosity problem of these natural oils, the fatty acids are removed from the glycerol backbone through transesterification (Figure 3). Transesterification occurs when triacylglycerols react with alcohol, most commonly methanol, and an acidic or alkaline catalyst (KOH, NaOH) to form 3 moles of fatty acid ethyl esters and 1 mole of glycerol.

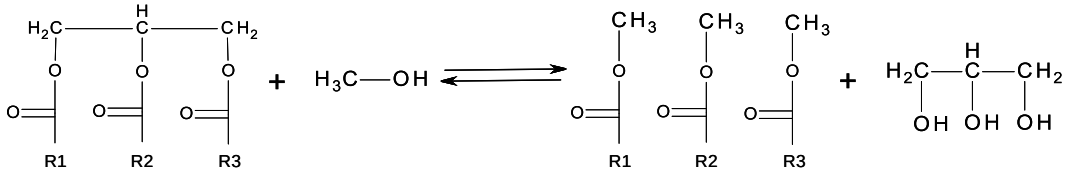


Figure 3. Transesterification Reaction. 3 moles of methanol react with 1 mole of triacylglycerol producing 3 moles of biodiesel (alkyl esters) and one mole of glycerol.

Crude glycerin is a by-product of biodiesel production and is mostly composed of glycerol (Figure 3). Aside from glycerol, crude glycerin also contains free fatty acids,

methanol, soap, and alkaline catalyst (KOH or NaOH) (Mu, Teng, Zhang, Wang, & Xiu, 2006). Depending on production processing, 55-90% (v/v) of crude glycerin is glycerol (80-85% on average). Crude glycerin is approximately 10% of the final product of transesterification; 1 pound generated per 10 pounds of biodiesel produced (Yazdani & Gonzalez, 2007). Crude glycerin can reach commercial quality through different purification methods, such as filtration, chemical additions, or fractional distillation; however, these processes are often not cost efficient (Pachauri & He, 2006). With the worldwide expansion of biodiesel production in recent years, crude glycerin has begun to flood the market, and although it is a common ingredient in many household products such as food, cosmetics, and pharmaceuticals, its value has dropped significantly (Yazdani & Gonzalez, 2007; Hájek & Skopal, 2010; Pyle, Garcia, & Wen, 2008). From 2004 to 2006, biodiesel production increased 10-fold which caused the price of crude glycerin to drop from $0.25 to $0.03-$0.05 per pound (Yazdani & Gonzalez, 2007; Pyle et al., 2008). Today, crude glycerin is considered a waste product and a problem that all biodiesel production companies are facing. Developing processes that can convert crude glycerin into higher value products will help transform a waste by-product into valued, green, alternative fuels. Many novel studies focusing on crude glycerin utilization are underway. Some of these studies include converting it into valuable products such as 1,3-propanediol, hydrogen gas, succinic acid, 1,2-propanediol, dihydroxyacetone, polyesters, polyglycerols, and polyhydroxyalkanoates (Pachauri & He, 2006; Voegele, 2010). The following study presents another pathway of utilization that could help alleviate the crude glycerin surplus by turning it into a profitable, renewable product; biogas. Through a

mixed consortium of sulfate-reducing bacteria (SRB) and methanogens, crude glycerin was biologically converted to methane.

Biogas. Natural gas is approximately 90% methane. Methane is one of the most energy efficient fuels because it releases more heat than other hydrocarbon fuels per carbon. It is also cleaner since it does not produce ash or residues when burned. The following equation represents the combustion of methane:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \quad (2)$$

The caloric value of methane combustion is 55,600 kJ/kg. This is greater than both petroleum and coal which have calorific values of 46,000 and 35,000 kJ/kg, respectively. The ratio of H:C bonds in methane is 4, while petroleum has a ratio of 2 and coal has one less than 1. In general, as the H:C atomic ratio decreases so does the calorific value (Schobert, 1990).

Microbial Physiology.

Physiology of SRB on Glycerol.

SRB live in anaerobic environments and produce hydrogen sulfide through the reduction of sulfate (SO_4^{2-}) via the oxidation of hydrogen and/or organic carbon. Different SRBs can either oxidize organics incompletely to acetate or completely to CO_2. (Muyzer & Stams 2008; Barton & Hamilton, 2007). Natural habitats of SRB include marine or freshwater sediments, soils, hot springs, geothermal environments, oil wells and animal gastrointestinal tracts (Pereira et al., 2008; Postgate, 1979). They have a large impact on carbon and sulfur cycling and have recently been found useful for biotechnological applications including heavy metal immobilization, wastewater treatment for sulfur removal, and hydrogen production. However, SRB can also cause economic problems that include water pollution through high sulfide production, metal and stone work corrosion, oil spoilage, and food spoilage (Pereira et al., 2008; Muyzer & Stams 2008).

SRB are not limited to sulfate for anaerobic respiration. Some are capable of reducing elemental sulfur, thiosulfate, fumarate, dimethylsulfoxide, Mn(IV), Fe(III), U(VI), Tc(VII), As(V), and Cr(VI) (Barton & Hamilton, 2007; Pereira et al., 2008). Aside from having the capability of reducing a wide range of terminal electron acceptors, SRB can also oxidize a variety of substrates such as lactate, hydrogen, acetate, ethanol, propionate, pyruvate, etc. (Pereira et al., 2008). Some SRB are also able to utilize glycerol, the main component of crude glycerin. Figure 4 represents a possible pathway of glycerol oxidation to acetate. Glycerol enters in glycolysis as glyceraldehyde-3-

phosphate (Temudo, Poldermans, Kleerebezem, & Loosdrecht, 2008). Glycerol kinase phosphorylates glycerol through the use of one molecule of ATP and then an FAD, bound to a glycerophosphate dehydrogenase, oxidizes the glycerol-3-phosphate to dihydroxyacetone phosphate. Triosephosphate isomerizes dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. Then glyceraldehyde-3-phosphate gets oxidized to pyruvate generating two molecules of ATP and one molecule of NADH (White, 2007). Anaerobically, either pyruvate-ferredoxin oxidoreductase or pyruvate-formate lyase oxidizes pyruvate to acetyl-CoA which then gets converted by phosphotransacetylase to acetyl phosphate (White, 2007; Temudo et al., 2008). The phosphate group of acetyl phosphate gets transferred to ADP via acetate kinase forming acetate and ATP (White, 2007; Temudo et al., 2008). The following reaction equation summarizes the products of incomplete glycerol oxidation with sulfate as the terminal electron acceptor (Barton & Hamilton, 2007):

$$C_3H_8O_3\,(\text{Glycerol}) + SO_4^{2-} + 3H^+ \rightarrow C_2H_4O_2\,(\text{Acetate}) + CO_2 + HS^- + 3H_2O \qquad (3)$$

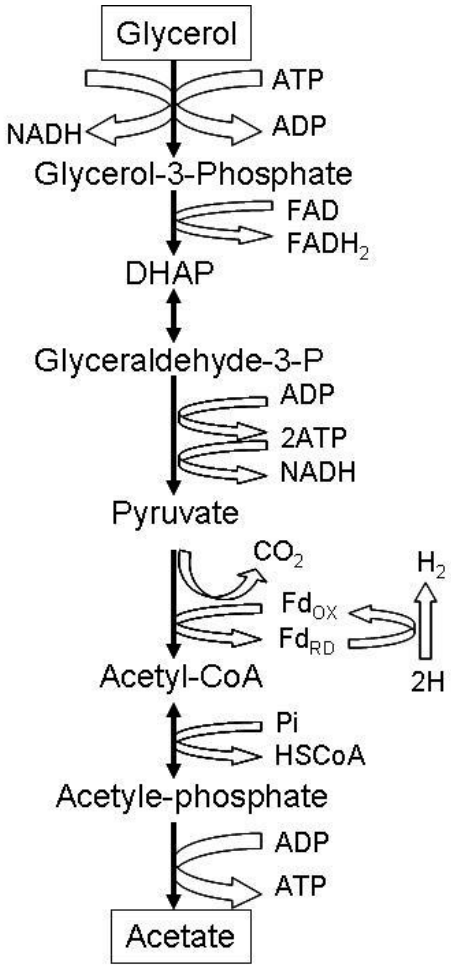


Figure 4. Incomplete Oxidation of Glycerol. Anaerobic fermentation pathway for glycerol degradation (Adapted from Temudo et al., 2008).

Physiology of Methanogenesis.

Methanogens flourish in anoxic habitats that contain low concentrations of terminal electron acceptors like sulfate, nitrate, and Fe(III) (Liu & Whitman, 2008). When high concentrations of these terminal electron acceptors are present, methanogens are outcompeted. It is more thermodynamically favorable for the other microorganisms to reduce these electron acceptors compared to methanogens reducing CO_2 to methane (Liu

& Whitman, 2008). Methanogens are at the bottom of the food chain in terms of metabolism of organic compounds. They reduce CO_2, the final end product of organic degradation, and produce about 1 billion tons of methane each year globally, playing a very important role in global carbon cycling (Ferry, 1994; Goenrich, Duin, Mahlert, Thauer, 2005). Methanogens are observed in many diverse anaerobic environments such as marine and freshwater sediments, flooded soils and peat bogs, landfills, animal gastrointestinal tracts, anaerobic digestors, tundra, rice fields and geothermal systems (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994). Methanogens can only utilize three types of substrates: CO_2, methylated compounds, and acetate (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994). With such a limited substrate metabolism, methanogens rely on other microorganisms to degrade larger and more complex organic compounds, leaving them with lower energy molecules to be used for growth (Liu & Whitman, 2008).

Hydrogenotrophic Methanogenesis. Hydrogenotrophic methanogens utilize CO_2 and H_2 as carbon and energy sources (see equation 4) (Liu & Whitman, 2008). Figure 5 shows the carbon pathway of hydrogenotrophic methane production.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (4)$$

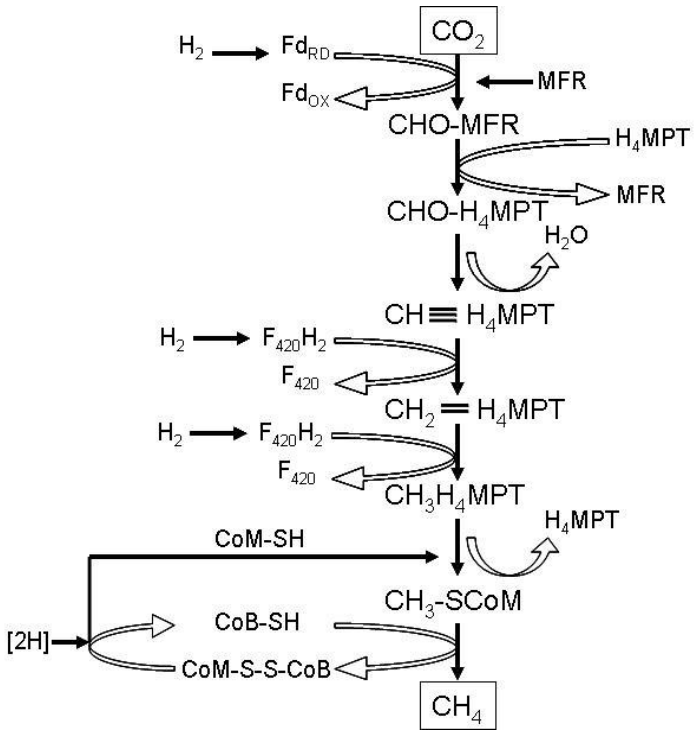


Figure 5. Hydrogenotrophic Metabolism (Adapted from Liu & Whitman, 2008).

As shown in Figure 5, CO_2 enters the methanogenic pathway and gets reduced to formyl-methanofuran (CHO-MFR) via formyl-MFR dhydrogenase. Methanofuran (MFR) is a carrier coenzyme that binds to reduced CO_2. The formyl group is then transferred to tetrahydromethanopterin (H_4MPT) forming the intermediate formyl-H_4MPT. The formyl group losses a water molecule and gets reduced to methyl-H_4MPT by a series of stepwise reductions using coenzyme $F_{420}H_2$ as a reducing agent. Next, the methyl- H_4MPT:HS-CoM methyltransferase transfers the methyl group to CoM-SH forming methyl-SCoM (methyl-mercaptoethanesulfonate). Coenzyme B then donates electrons so methyl coenzyme M reductase can cleave and reduce the methyl group to methane. The heterodisulfide, CoM-S-S-CoB, forms after the cleavage and reduction of the methyl group and gets reduced by the heterodisulfide reductase. For every one molecule of CO_2

and four molecules of H_2 entering the pathway, one molecule of methane gets generated (Liu & Whitman, 2008; Deppenmeier, 2004).

Methylotrophic Methanogenesis. Methylated compounds, such as methanol and formate, are metabolized via the methylotrophic pathway producing CO_2 and CH_4 as end products (see equation 5). Figure 6 shows the predicted pathway for methane production when methanol is the main substrate (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994). For every four methyl groups entering the pathway, one gets oxidized to CO_2 while the remaining three get reduced to methane (Deppenmeier, 2004). In the reduction pathway, the methyl group of methanol gets transferred via substrate-specific methyltransferase to a corrinoid protein and then to CoM-SH forming methyl-SCoM. The methyl group is then reduced to methane through the same pathway as hydrogenotrophic CO_2 reduction (Figures 5 and 6). In the oxidative pathway, methyl-SCoM follows the reverse pathway of hydrogenotrophic CO_2 reduction (Figures 5 and 6) so that the methyl group eventually gets oxidized to CO_2. From this oxidation pathway, reducing equivalents for methane production are generated (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994).

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad (5)$$

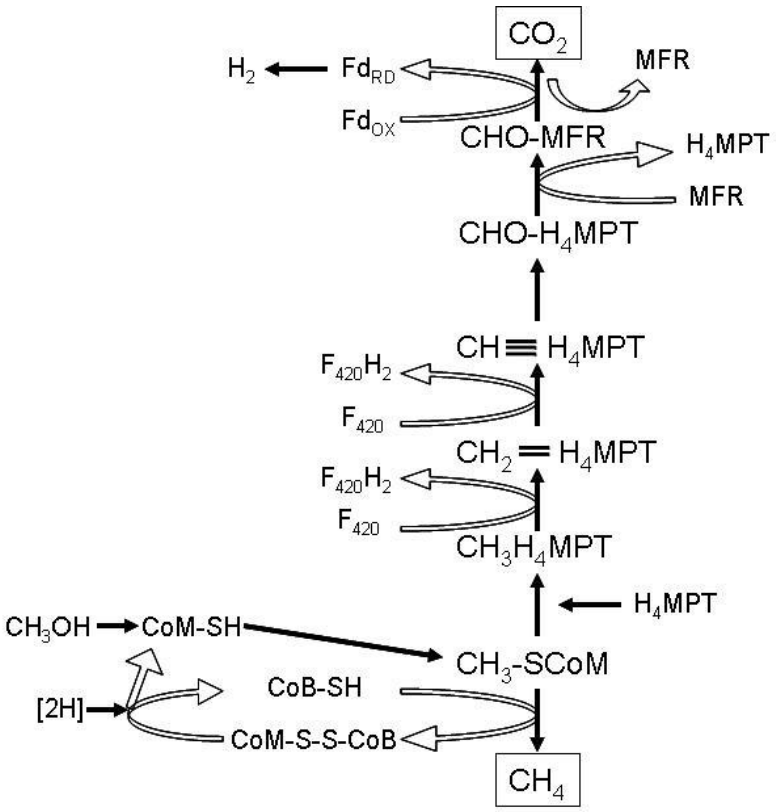


Figure 6. Methylotrophic Pathway (Adapted from Liu & Whitman, 2008).

Acetoclastic Methanogenesis. The third type of substrate utilized by methanogens is acetate. Although only *Methanosarcina* and *Methanosaeta* are capable of metabolizing acetate, the majority of methane produced biologically comes from acetoclastic (acetate eating) methanogens (Liu & Whitman, 2008). In this metabolism (see Figure 7), acetate gets split into CO_2, through carboxyl oxidation, and CH_4, through methyl reduction (see equation 6) (Liu & Whitman, 2008; Deppenmeier, 2004; Lovely, Dwyer, & Klug, 1982). *Methanosarcina* and *Methanosaeta* each have different acetoclastic pathways containing their own unique enzymes (Liu & Whitman, 2008). The first step of methanogenesis

involves the activation of acetate through phosphorylation. The acetyl group is then transferred to SH-CoA (coenzyme A) via acetate kinase-phoshotransacetylase (*Methanosarcina*) or AMP-forming acetyl-CoA synthase (*Methanosaeta*) producing acetyl-CoA (CH_3-CO-S-CoA) (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994). Then, CO dehydrogenase (CODH) cleaves the acetyl-CoA complex. After cleavage, the methyl group gets transferred to H_4MPT and then follows the methylotrophic (Figure 6) and hydrogenotrophic (Figure 5) pathways to methane formation. The cleaved CO group gets oxidized to CO_2 which generates electrons that help drive methane production (Liu & Whitman, 2008; Deppenmeier, 2004; Ferry, 1994).

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad (6)$$

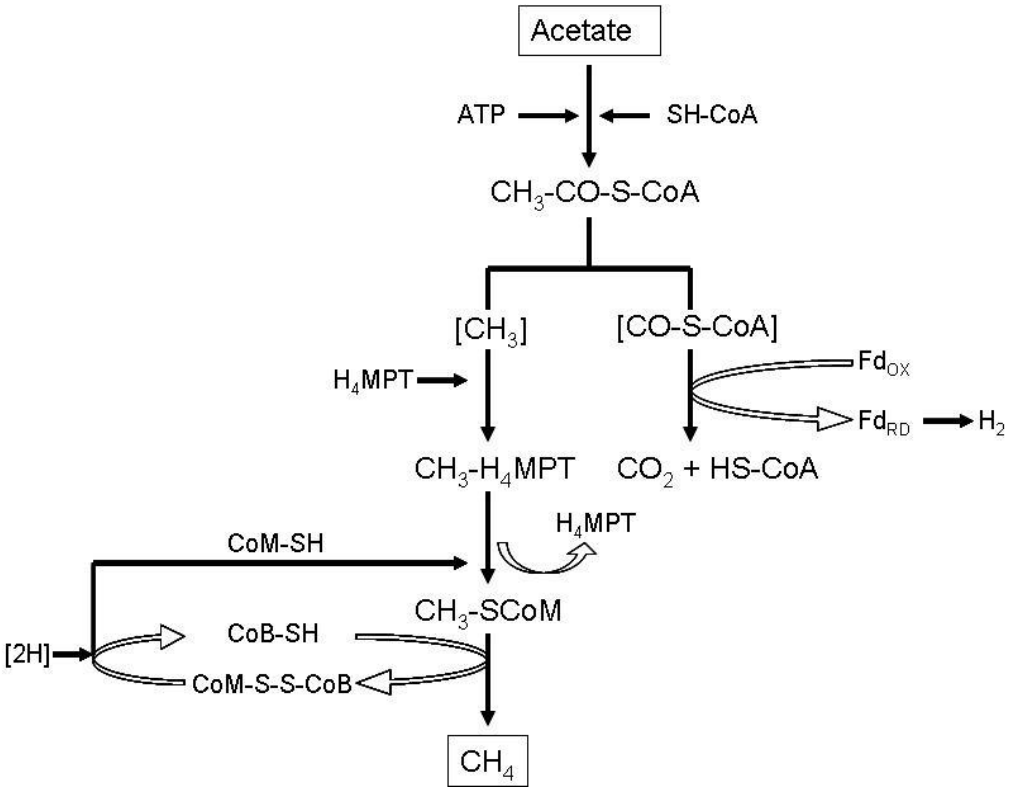


Figure 7. Acetoclastic Pathway (Liu & Whitman, 2008).

Syntrophic Growth of SRB and Methanogens.

As described previously, SRB can utilize organic molecules, like lactate and glycerol, as sole energy sources to reduce sulfate to hydrogen sulfide. However, sulfate is not a thermodynamically favorable electron acceptor. It has to be activated to adenosine-phosphosulphate (APS) by an ATP sulphurylase in order for oxidation to proceed (See equation 7). In order to overcome the thermodynamically unfavorable generation of PP_i, which happens through the formation of APS, the enzyme, pyrophosphatase, must also hydrolyze PP_i to $2P_i$ (See equation 8). Two ATP molecules are used during the activation of sulfate (Muyzer & Stams, 2008; Zehnder, 1988).

$$SO_4^{2-} + ATP \rightarrow APS + PP_i \quad (7)$$

$$H_2O + PP_i \rightarrow 2P_i \quad (8)$$

As substrate oxidation occurs, electrons get transferred (by an unknown carrier) to a cytoplasm facing, membrane-bound, ferridoxin-specific, Coo hydrogenase or Ech hydrogenase (dependant upon carbon source). Within the hydrogenase, electrons get transferred onto protons generating H_2 which then diffuses out of the cytoplasm into the periplasm (Barton & Hamilton, 2007; White, 2007; Pereira et al., 2008). When H_2 enters the periplasm, it gets re-oxidized to $2H^+$ by a periplasmic cytochrome c_3 hydrogenase. The protons go to the outside of the membrane creating a transmembrane proton gradient which can then drive ATP synthesis. The electrons move back across the membrane reducing sulfate to sulfide. (Zenhnder, 1988; Barton & Hamilton, 2007; Pereira et al., 2008). Hydrogen cycling is the name of this process; H_2 is generated in the cytoplasm and then oxidized in the periplasm (Barton & Hamilton, 2007; Walker et al., 2009; Caffery et al., 2007).

When sulfate is not present hydrogen gets produced instead of hydrogen sulfide (Barton & Hamilton, 2007):

$$C_3H_8O_3 + H_2O \rightarrow C_2H_4O_2 + CO_2 + 3H_2 \quad (9)$$

Without a terminal electron acceptor, H_2 increases and the Coo hydrogenase is unable to re-oxidize the unknown electron carrier thus inhibiting SRB growth (Walker et al., 2009). This is problematic since the electrons have nowhere to go besides H_2. In order for

organic oxidation to continue, the H_2 partial pressure needs to remain low (~10-100 Pa) so a thermodynamically favorable environment can be maintained (Barton & Hamilton, 2007; Walker et al., 2009; Bryant, Campbell, Reddy, & Crabill, 1977; Ishii, Kosaka, Hori, Hotta, & Watanabe, 2005). Through a process termed interspecies hydrogen transfer, methanogens can grow syntrophically with SRB. Methanogens can utilize the H_2 product built up as an energy source for growth. By doing so, they create a low H_2 partial pressure environment which is thermodynamically favorable for SRB to continue oxidizing organic compounds (Walker et al., 2009; Valdez-Vazquez & Poggi-Varaldo, 2009; Stams & Plugge, 2009; Hamilton, 1998).

SRB and methanogens can both utilize H_2 and acetate as energy sources, and in the natural environment, they compete for these metabolic precursors (Sonne-Hansen, Westermann, & Ahring, 1999; McCartney & Oleszkiewicz, 1993). When grown on acetate and H_2, SRB K_m values are 0.2 mM and 0.001 mM, respectively. Methanogens have higher K_m values for both compounds: 3.0 mM on acetate and 0.006 mM on H_2 (Isa, Grusenmeyer, & Verstraete, 1986). Since SRB have higher affinities (lower K_m values) for these substrates they are capable of utilizing them more efficiently, keeping concentrations too low for methanogenic growth to proceed (Sonne-Hansen et al., 1999; Isa et al., 1986; Lovely et al., 1982; McCartney & Oleszkiewicz, 1993; Lovley & Phillips, 1987). SRB only compete with methanogens when terminal electrons are present. Even at millimolar concentrations of sulfate, SRB will outcompete methanogens for H_2 and acetate in the presence of SO_4^{2-} (Isa et al., 1986; Lovely et al., 1982). In environments where terminal electron acceptors are limited or as they become exhausted,

syntrophic growth between SRB and methanogens will begin to take place (Vroblesky, Bradley, & Chapelle, 1996). In the following study, different SRB and methanogen co- and triculture combinations were developed for the utilization of glycerol and conversion to methane.

Experimental Microorganisms.

Desulfovibrio alcoholvorans 6133.

D. alcoholvorans 6133 is an anaerobic, Gram negative, sulfate reducing bacterium that was isolated from an industrial anaerobic fermenter containing waste water from an alcohol distillery. *D. alcoholvorans 6133* is curved, non-spore forming, and motile (single polar flagellum). When utilizing glycerol as a carbon source, it oxidizes it incompletely to acetate. *D. alcoholvorans 6133* can also incompletely oxidize a variety of other compounds including dihydroxyacetone, 1,3-propanediol, ethanol, lactate, pyruvate etc. This organism is also capable of oxidizing methanol and formate to carbon dioxide (Qatibi, Niviere, & Garcia, 1991).

Desulfovibrio vietnamensis.

D. vietnamensis is an anaerobic, Gram negative, sulfate reducing bacterium that was isolated from a crude oil storage tank in Southern Vietnam near the town Vung Tau. This organism is curved shaped, non-spore forming, and prefers to grow in the presence of 5% NaCl. In the presence of sulfate, *D. vietnamensis* can utilize a large range of carbon sources including, lactate, malate, formate, ethanol, and glycerol (Nga, Ha, Hien, & Stan-Lotter, 1996; Feio et al., 2004).

Methanococcus maripaludis.

M. maripaludis is a strictly anaerobic organism that was isolated in the 1980s from salt marsh sediments in Pawley's Island, North Carolina. The name *maripaludis* means "of the sea marsh". This hydrogenotrophic methanogen is non-spore forming, has weak motility, and has cell walls that do not contain muramic acid. *M. maripaludis* is a coccoidal-rod about 1.2 by 1.6 μm in length, is mesophilic, and displays optimal growth in the presence of 100 mM NaCl. (Walters & Chong, 2009; Jones, Paynter, & Gupta, 1983).

Methanoculleus marisnigri.

M. marisnigri was isolated in sediment from the Black Sea. This organism is an irregular coccoid and cells are about 1 to 2 μm in diameter growing singly or as pairs. It is capable of growing on H_2/CO_2, formate and some secondary alcohols. M. *marisnigri* grows best at neutral pH, mesophilic temperatures, and at NaCl concentrations lower than 300 mM. Although, the organism has flagella, it has not demonstrated motiliy (ExPASy Proteomics Server; Maestrojuán, Boone, Xun, Mah, & Zhang, 1990; Anderson et al., 2009).

Methanosarcina acetivorans.

M. acetivorans is an acetoclastic methanogen isolated from methane-evolving marine sediments in Scripps Canyon near La Jolla, CA. The cells are irregularly shaped cocci and have a diameter of about 1.9 μm. When grown on acetate, *M. acetivorans* grows singly during early exponential-phase, but as it reaches late stationary-phase, it

forms aggregates of 2 to 12 cells. This organism has a single fimbria-like appendage but motility has not been observed. *M. acetivorans* is a metabolically versatile methanogens that can utilize acetate, mono-methylamine, di-methylamine, tri-methylamine, methanol, or carbon monoxide as sole carbon and energy sources. Cells do not display growth on H_2/CO_2. The organism requires both NaCl and $MgSO_4$ for growth, and achieves optimal growth with NaCl concentrations between 100 and 600 mM. This is the first known marine isolate that is capable of utilizing acetate as a carbon and energy source (Sowers, Baron, & Ferry, 1984; Rohlin & Gunsalus, 2010; Ma, Liu, & Dong, 2006).

Thesis Statement and Hypothesis.

Glycerin is an abundant waste product from biodiesel production. Converting glycerin into a valuable product, like methane, will help improve biodiesel economics. The goal of this study was to characterize the syntrophic associations between SRB and hydrogenotrophic and acetoclastic methanogens and their ability to convert crude glycerin into methane. Converting glycerin into methane through microbial conversion is predicted to display high yields and a promising value stream for the waste product.

MATERIALS AND METHODS

Glycerin Production and Fractionation.

Biodiesel.

44 grams of methanol and 2 grams of KOH were added to 200 grams of soybean oil (Su-per Soybean Oil, Gateway Products Incorporated). The mixture was heated to 68˚C for 1 hour. The biodiesel/glycerin end-product was allowed to cool and separate for 24 hours. After 24 hours the two phases were separated into serum bottles via pipetting. Crude glycerin was degassed with N_2 gas for 1 hour, sealed, and then autoclaved.

Fractionated Glycerin.

50 ml of crude glycerin was mixed in a 2:1 ratio with phosphoric acid and fractionated into 3 layers: free fatty acid, glycerol/methanol (fractionated glycerin), and potassium phosphate (catalyst precipitate). The free fatty acid and the fractionated glycerin layers were pipetted into separate serum bottles, degassed with N_2 gas for 1 hour, and then autoclaved (Addison, 2007).

Growth Experiments.

Strains and Culture Conditions.

Desulfovibrio alcoholvorans 6133, Desulfovibrio vietnamensis, Methanoculleus marisnigri, and *Methanosarcina acetivorans* wild-type cells were obtained from the German Resource Centre for Biological Material (DMSZ, Germany). *Methanococcus maripaludis* wild-type cells were obtained from the Stahl lab at the University of

Washington. Batch mono-, co-, and tricultures of each strain were grown anaerobically in serum bottles containing coculture medium (CCM). All cultures were grown under 20% CO_2 and 80% N_2 headspace with the exception of *M. marisnigri* and *M. maripaludis* monocultures, which were given 20% H_2 and 80% CO_2 headspace. Cultures were grown in anaerobic tubes (10 ml liquid volume with 15 ml headspace; 5 ml liquid volume with 20 ml headspace) or serum bottles (50 ml liquid volume with 75 ml headspace). CCM is a defined medium containing approximately 37mM NaCl, 27 mM $MgCl_2$ x $6H_2O$, 0.95 mM $CaCl_2$ x $2H_2O$, 1.1mM K_2HPO_4, 3.98µM Resazurine, 9.3 mM NH_4Cl, 4.5 mM KCl, 1 mM L-cysteine x HCl, 59.5 mM $NaHCO_3$, 1 mM trace minerals (Widdel et al. 1983), 1 mM Thauer's vitamins (Clark et al., 2007), 1mM Selenite-tungstate solution (Widdel & Banks, 1992), 1 mM Na_2S x $9H_2O$, and 50 mM Na_2SO_4 (*Desulfovibrio alcoholvorans 6133* and *Desulfovibrio vietnamensis* monocultures only) (pH 7.2). Unless otherwise stated, CCM serum bottles were inoculated with a 10% (vol/vol) inoculum of mid-log phase batch cultures. All cultures were grown in duplicate and incubated at 30° C.

SRB Monoculture Experiments.

Monocultures (10 ml final volume) of *D. alcoholvorans 6133* and *D. vietnamensis* were grown on 60 mM sodium lactate, 36 mM pure glycerol, 57 mM fractionated glycerin (based on glycerol concentration), and 37 mM crude glycerin (based on glycerol concentration). Growth was measured at various time points using a spectrophotometer (Spectronic 20D+, Thermo Electron Corporation, Waltham, MA) at a wavelength of 600 nm. Uninoculated CCM was used as a blank.

Monocultures of *D. alcoholvorans 6133* and *D. vietnamensis* were grown in 50 ml of CCM with 37 mM crude glycerin (based on glycerol concentration). 1 ml samples were collected aseptically via septum throughout growth. Growth was measured by UV-Vis spectrometer (Shimadzu UV-1700 PharmaSpec, Kyoto, Japan) at a wavelength of 600 nm. Samples were centrifuged (Beckman Coulter Microfuge® 16 Microcentrifuge, Brea, CA) at 14,500 RPM for 5 min at room temperature and the supernatant was stored at -20°C until analysis. The acetate concentration of the supernatant was determined for each sample collected.

Methanogen Toxicity Tests.

Cultures of *M. marisnigri, M. maripaludis,* and *M. acetivorans* were grown in approximately 5 ml of CCM. *M. marisnigri* and *M. maripaludis* were grown on 20% H_2 and 80% CO_2. *M. acetivorans* was grown on 150 mM methanol (normal conditions). Cultures were grown in the presence of increasing concentrations of crude glycerin: 0 mM, 13.7 mM, 22.8 mM, 37 mM (based on glycerol concentration) to measure toxicity. Methane concentration was determined at the end of growth.

Co- and Triculture Experiments.

Co- and tricultures were grown in sodium sulfate free CCM. Mid-log phase monocultures of *D. alcoholvorans 6133, D. vietnamensis*, *M. marisnigri,* and *M. maripaludis* were combined into six different culture combinations: *D. alcoholvorans 6133* and *M. maripaludis*; *D. vietnamensis* and *M. maripaludis*; *D. alcoholvorans 6133* and *M. marisnigri*; *D. vietnamensis* and *M. marisnigri; D. alcoholvorans 6133, M.*

maripaludis, and *M. marisnigri; D. vietnamensis, M. maripaludis,* and *M. marisnigri.* Each culture was grown in 10 and 50 ml of CCM. Cultures were given the same concentrations of pure glycerol, fractionated glycerin, and crude glycerin as described previously. Growth was measured and samples were collected as described previously. Optical density was measured from both 10 and 50 ml cultures. Methane was determined for the 10 ml cultures. Glycerol, sodium acetate, and free fatty acid concentrations were determined for the 50 ml cultures.

Glycerol Optimization Experiments.

10 ml cocultures of *D. alcoholvorans 6133* & *M. maripaludis* and *D. vietnamensis* & *M. maripaludis* were grown on 14, 36, 50, and 71 mM pure glycerol and 16, 41, 57, and 83 mM fractionated glycerin (based on glycerol concentration). Growth was measured via optical density. Methane and glycerol concentrations were measured at the end of growth.

Growth of *M. acetivorans* on Different Headspace Concentrations.

M. acetivorans was grown on 120 mM sodium acetate under the following N_2%:CO_2% headspaces: 100:0, 67:33, 50:50, 33:67. For a control, *M. acetivorans* was grown on 150 mM methanol under normal headspace (80:20) and under 100 % N_2 headspace. Growth was measured via optical density and methane concentrations were determined at late-stationary phase.

M. acetivorans Triculture Experiments.

M. acetivorans was combined at two different concentrations (initial ODs at approximately 0.04-0.05 and 0.08-0.1) with the following four cocultures: *D. alcoholvorans 6133* & *M. maripaludis*; *D. vietnamensis* & *M. maripaludis*; *D. alcoholvorans 6133* & *M. marisnigri*; *D. vietnamensis* & *M. marisnigri* (initial OD of approximately 0.08-0.1). Tricultures were grown on 37 mM crude glycerin and 33 mM methanol (based on glycerol concentrations, methanol from crude glycerin). Growth was measured via optical density and methane concentrations were determined at late-stationary phase.

Supernatant Growth Experiments**.**

M. acetivorans was grown on 150 mM methanol and 37 mM crude glycerin (based on glycerol concentration; 33 mM methanol present in this concentration of crude glycerin). Growth was measured via optical density and methane concentration was measured at late-stationary phase. Cultures were centrifuged (Hermle Z300K Labnet, Edison, NJ) for 10 minutes at 4,500 RPM. Supernatant was filtered using a 25 mm 0.2 µm syringe filter and transferred into anaerobic tubes via septum. *D. alcoholvorans 6133* & *M. maripaludis*, *D. vietnamensis* & *M. maripaludis*, *D. alcoholvorans 6133* & *M. marisnigri*, and *D. vietnamensis* & *M. marisnigri* were inoculated into filtered supernatant. Growth was measured via optical density and methane concentration was determined at late-stationary phase.

Data Analysis.

Glycerol Assay.

Glycerol concentrations were determined enzymatically using a Megazyme kit for glycerol (Megazyme International Ireland Ltd.).

Free Fatty Acid Assay.

Free Fatty Acid Quantification Kit (BioVision Research Products, Mountain View, CA) was used to determine the initial and final concentrations of free fatty acids from the 50 ml co- and tricultures grown on fractionated and crude glycerin.

Ion Chromatography.

Acetate concentrations were determined by ion chromatography (Dionex) with a 4mm IonPac® AS10A hydroxide-selective anion-exchange column.

Gas Chromatography .

Methane concentrations were determined by gas chromatography (Hewlett Packard Series II gas chromograph) using a Carboxen^{TM} 1000 column length, N_2 carrier gas (33-34 psi), and a thermal conductivity detector (TCD). The GC injection, oven, and detector temperatures were 200°C, 170°C, and 250°C, respectively.

Methanol concentrations of fractionated and crude glycerin were determine by gas chromatography (Hewlett Packard 5890 Series II Gas Chromotography) using a Tenax®-TA column (Alltech Associates, Inc., Deerfield, IL), at 190°C and 220°C using He as the

carrier gas (30, 40, 50 psi, respectively), and H_2 and air was used for the flame ionization detector (FID).

RESULTS

Monoculture Growth Experiments.

SRB Monocultures.

Both SRB monocultures, *D. vietnamensis* and *D. alcoholvorans 6133*, were capable of growing on lactate, pure glycerol, fractionated glycerin, and crude glycerin under sulfate-reducing conditions (Figure 8A-D, Table 1). *D. vietnamensis* grew faster than *D. alcoholvorans 6133* on all 4 carbon sources and both displayed the highest growth rate on lactate (0.053±0.004 and 0.031±0.004 h^{-1}, respectively). *D. vietnamensis* grew the slowest on crude glycerin (0.029 ±0.001 h^{-1}) while *D. alcoholvorans 6133* grew the slowest on pure glycerol and fractionated glycerin (0.014±0.001h^{-1} and 0.014±0.004h^{-1}, respectively). *D. alcoholvorans 6133* displayed a 43 hour lag when grown on fractionated glycerin *D. vietnamensis* grew faster on pure glycerol (0.028±0.002 h^{-1}) than fractionated glycerin (0.023±0.001 h^{-1}). Although *D. vietnamensis* grew faster on all carbon sources, *D. alcoholvorans 6133* reached a higher optical density when growing on lactate and crude glycerin. Since crude glycerin particulates can interfere with optical density readings, acetate production was also measured to track growth (Figure 8C-D). *D. vietnamensis* and *D. alcoholvorans 6133* produced approximately 39.78±1.812 and 19.63±0.755 mM of acetate, when grown on 37 mM crude glycerin. The results indicated that both *Desulfovibrio* species utilized glycerol at a slower rate than lactate, *D. vietnamensis* grew faster, but *D. alcoholvorans* obtained higher biomass numbers.

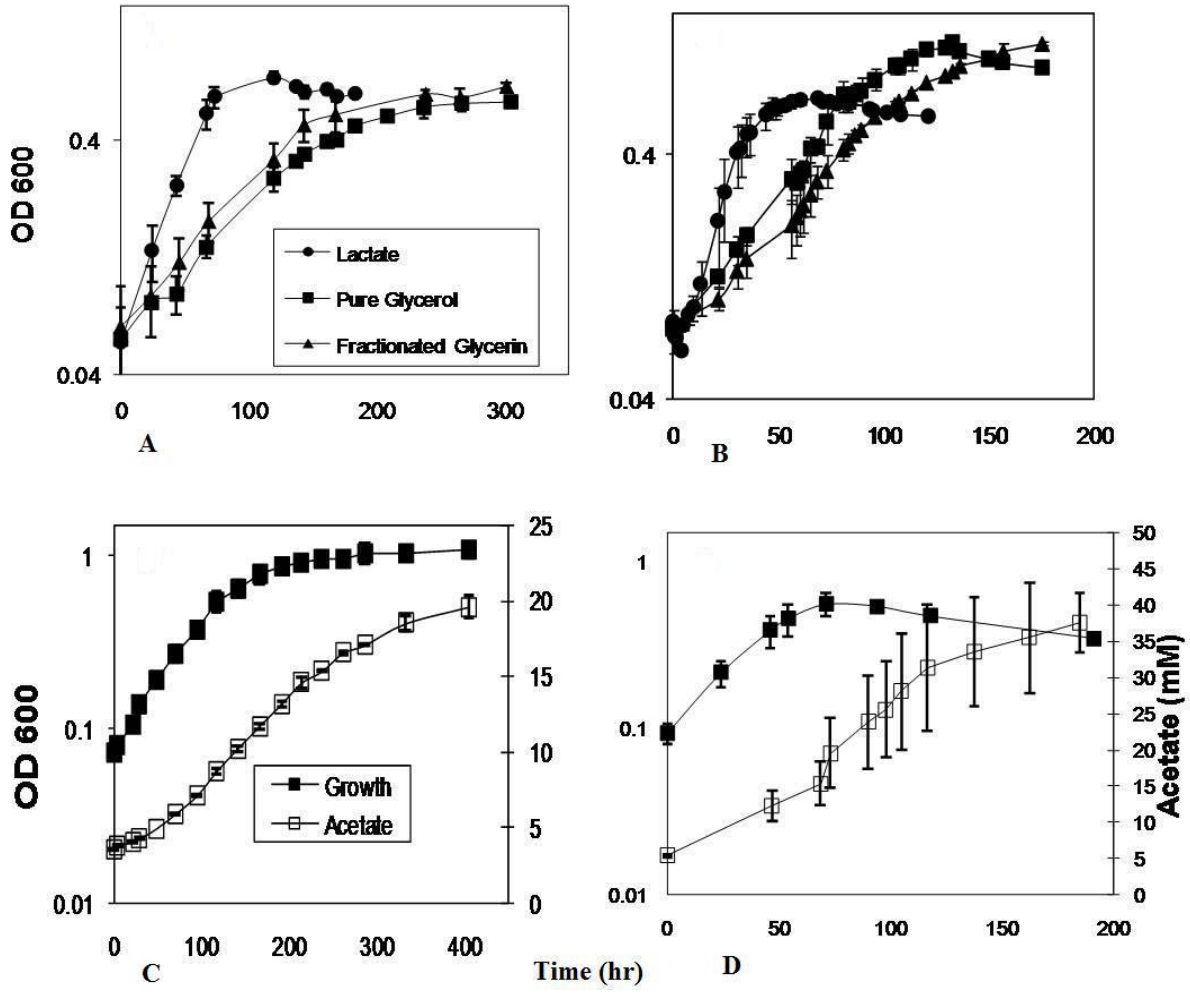


Figure 8. SRB Monoculture Growth on Different Carbon Sources. **(**A) *D. alcoholvorans 6133* and (B) *D. vietnamensis*. Cultures grown on 60 mM sodium lactate, 36 mM pure glycerol, or 57 mM (0.07 ml) fractionated glycerin (based on glycerol concentration) in the presence of 50 mM sodium sulfate. (C) *D. alcoholvorans 6133* and (D) *D. vietnamensis* cultures grown on 37 mM (0.08 ml) of crude glycerin (based on glycerol concentration) in the presence of 50 mM sodium sulfate. Data points are averages of two independent biological experiments (n=2).

Table 1. SRB Growth Rates on Different Carbon Sources.

Culture	Growth Rates (h^{-1})			
	Lactate	**Pure Glycerol**	**Fractionated Glycerin**	**Crude Glycerin**
D. alcoholvorans 6133	0.031±0.004	0.014±0.001	0.014±0.004	0.015±0.001
D. vietnamensis	0.053±0.004	0.028±0.002	0.023±0.001	0.029±0.001

Methanogen Monoculture Toxicity Experiments.

Because known methanogens cannot utilize glycerol, all methanogenic cultures required the presence of a non-methanogen glycerol-oxidizer (*i.e.*, SRB). Therefore, the production of methane in the presence of increasing amounts of crude glycerin was determined because methanogen growth could be inhibited by glycerol and/or contaminants present in crude glycerin (Figures 9-11). *M. maripaludis*, *M. marisnigri*, and *M. acetivorans* could produce methane in the presence of increasing levels of crude glycerin. *M. maripaludis* produced approximately 11.4±0.5 mM CH_4 when grown in the presence of 0, 14, 23, and 37 mM crude glycerin (based on glycerol concentration) with CO_2 and H_2 as carbon and energy sources. *M. marisnigri* produced an average concentration of 18.5 ±1.0 mM CH_4 under the same conditions in the presence of 0, 14, and 23 mM crude glycerin. However, the methane production rate of *M. marisnigri* declined when the glycerin levels were above 23 mM and methane was not produced in the presence of 37 mM crude glycerin. When grown on 150 mM methanol in the presence of the same concentrations of crude glycerin, *M. acetivorans* produced

approximately 99±22 mM CH_4. These results indicated that methane production was not inhibited by the presence of potential contaminants in glycerin for the tested methanogens when grown as monocultures with respective preferred substrates.

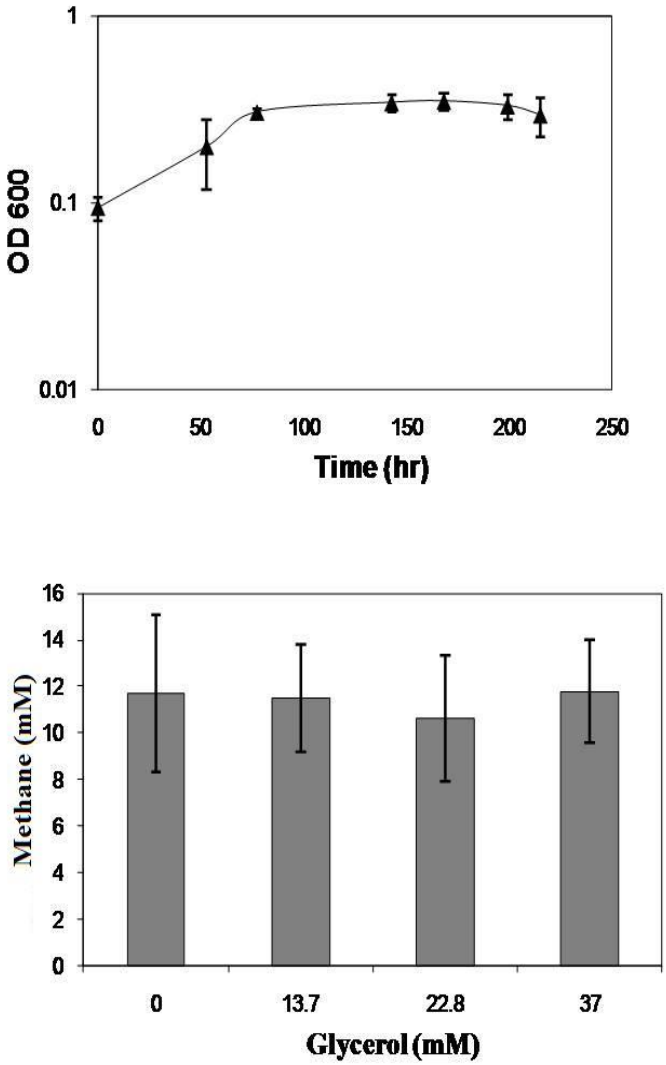
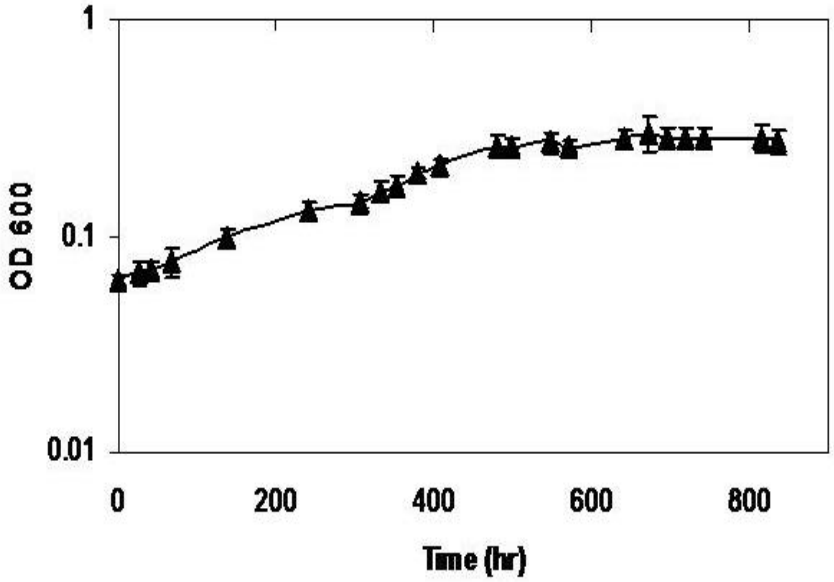


Figure 9. *M. maripaludis* Toxicity Test. (A) *M. maripaludis* grown on 20% H_2: 80% CO_2 (no glycerin present). (B) *M. marisnigri* grown on 20% H_2: 80% CO_2 in the presence of different concentrations of crude glycerin (based on glycerol concentration). Methane concentrations were determined when the *M. maripaludis* culture in the absence of crude glycerin reached stationary phase. Data points and experimental values are averages of two independent biological experiments (n=2).



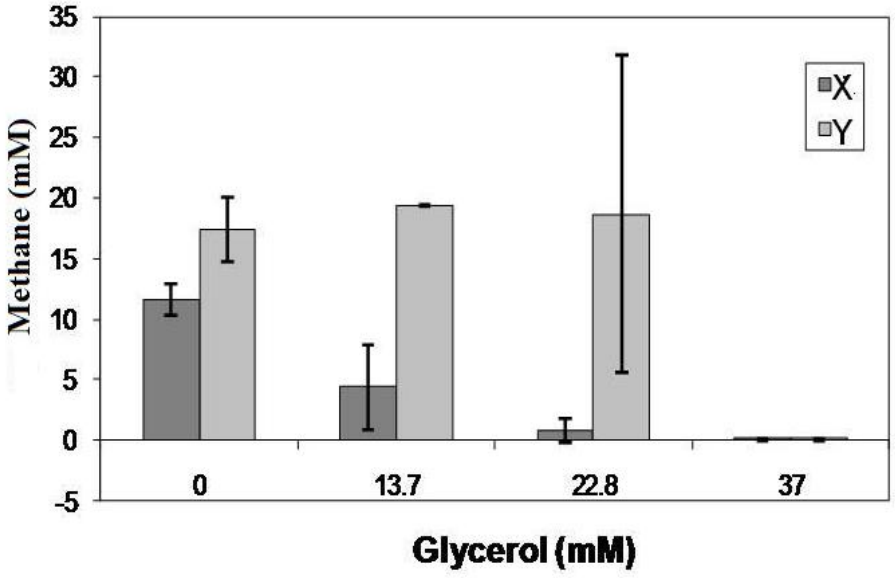


Figure 10. *M. marisnigri* Toxicity Test. (A) Growth of *M. marisnigri* on 20% H_2: 80% CO_2 (no glycerin present). (B) *M. marisnigri* grown on 20% H_2: 80% CO_2 in the presence of different concentrations of crude glycerin (based on glycerol concentrations). X methane concentration at stationary phase, Y methane concentration 2 weeks after stationary-phase. Data points and experimental values are averages of two independent biological experiments (n=2).

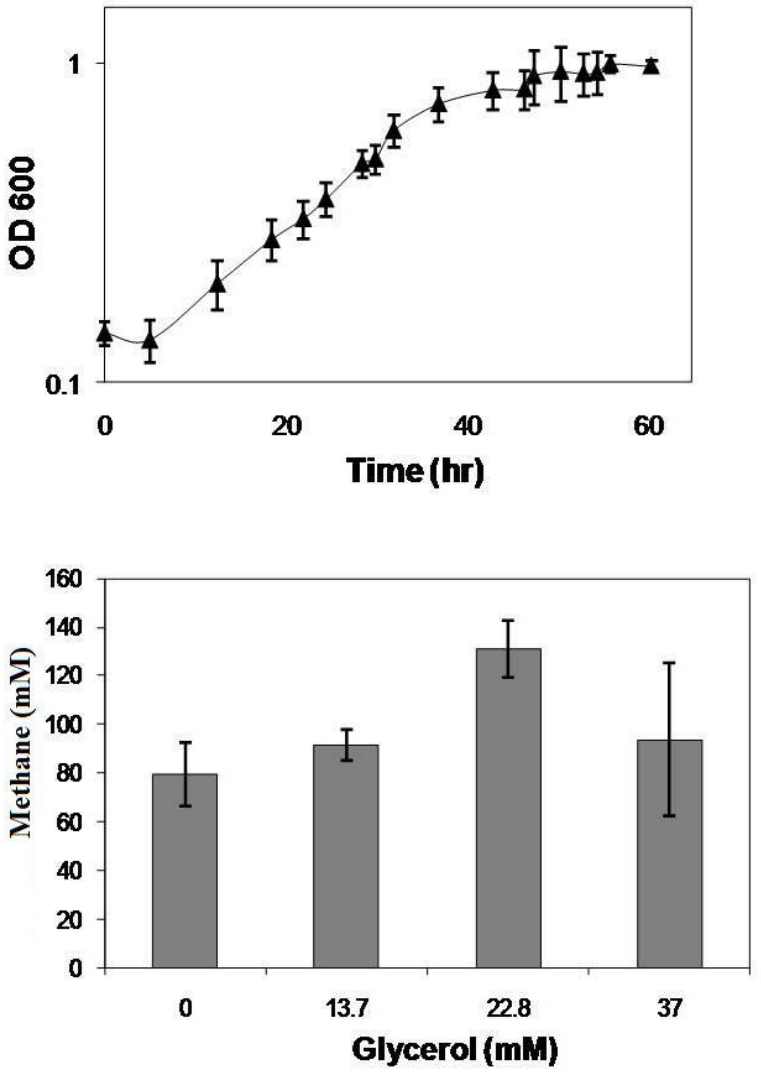


Figure 11. *M. acetivorans* Toxicity Test. (A) Growth of *M. acetivorans* on 150 mM methanol under 20% CO_2: 80% N_2 headspace (no glycerin present). (B) *M. acetivorans* grown on 150 mM methanol in the presence of different concentrations of crude glycerin (based on glycerol concentrations). Data points and experimental values are averages of two independent biological experiments (n=2).

Pure Glycerol and Fractionated Glycerin Optimization for Cocultures.

Pure Glycerol.

Cocultures of *D. vietnamensis* and *M. maripaludis* or *D. alcoholvorans 6133* and *M. maripaludis* were capable of growing with 14, 37, 50 and 71 mM of pure glycerol and 16, 41, 57, and 82 mM of fractionated glycerin (based on glycerol concentration) (Figures 12 & 13). When the concentration of pure glycerol was varied, the growth rates or final biomass yields were not affected for either coculture. The *D. vietnamensis* and *M. maripaludis* cocultures consumed approximately 30 mM of pure glycerol and produced approximately 20 mM of methane (Figure 13, Table 2). For the *D. alcoholvorans 6133* and *M. maripaludis* cocultures, glycerol remained for the three highest glycerol levels at the end of growth (Figure 12, Table 2). *D. alcoholvorans 6133* and *M. maripaludis* utilized a maximum of 41 mM glycerol and produced about 16.5 mM CH_4. For both cocultures, glycerol utilization increased as glycerol concentration increased, but methane production was not directly related to the amount utilized. Although *D. vietnamensis* and *M. maripaludis* consumed higher concentrations of glycerol when grown on 50 and 71 mM, D. *alcoholvorans 6133* and *M. maripaludis* produced more methane (Table 2) (17.0±6.0 and 16.5±1.3 mM CH_4, respectively versus 12.0±4.5 and 12.9±4.3 mM CH_4, respectively).

Fractionated Glycerin.

In contrast to pure glycerol, varying the concentration of fractionated glycerin affected growth rates. When grown on 82 mM fractionated glycerin D. *alcoholvorans*

6133 and *M. maripaludis* experienced a lag phase of approximately 100 hours and slower growth compared to growth on the other three lower concentrations of fractionated glycerin (Figure 12). Although growth rate did not decline, *D. vietnamensis* and *M. maripaludis* displayed a lag phase of approximately 70 to 100 hours on all concentrations of fractionated glycerin with the exception of the 16 mM culture (Figure 13). Cocultures of *D. vietnamensis* and *M. maripaludis* utilized approximately 30 mM fractionated glycerin; however, the coculture grew faster with 16 mM fractionized glycerin versus the higher concentrations of crude glycerin. Similar amounts of methane were produced (approximately 8.5 mM) by *D. vietnamensis* and *M. maripaludis* when grown on 16 and 41 mM fractionated glycerin. Although the growth of *D. alcoholvorans 6133* and *M. maripaludis* was slower on fractionated glycerin, the coculture of *D. alcoholvorans* and *M. maripaludis* was not as sensitive compared to coculture with *D. vietnamensis*. *D. alcoholvorans* and *M. maripaludis* utilized approximately 30 mM fractionated glycerin producing approximately 17 mM methane (Table 3).

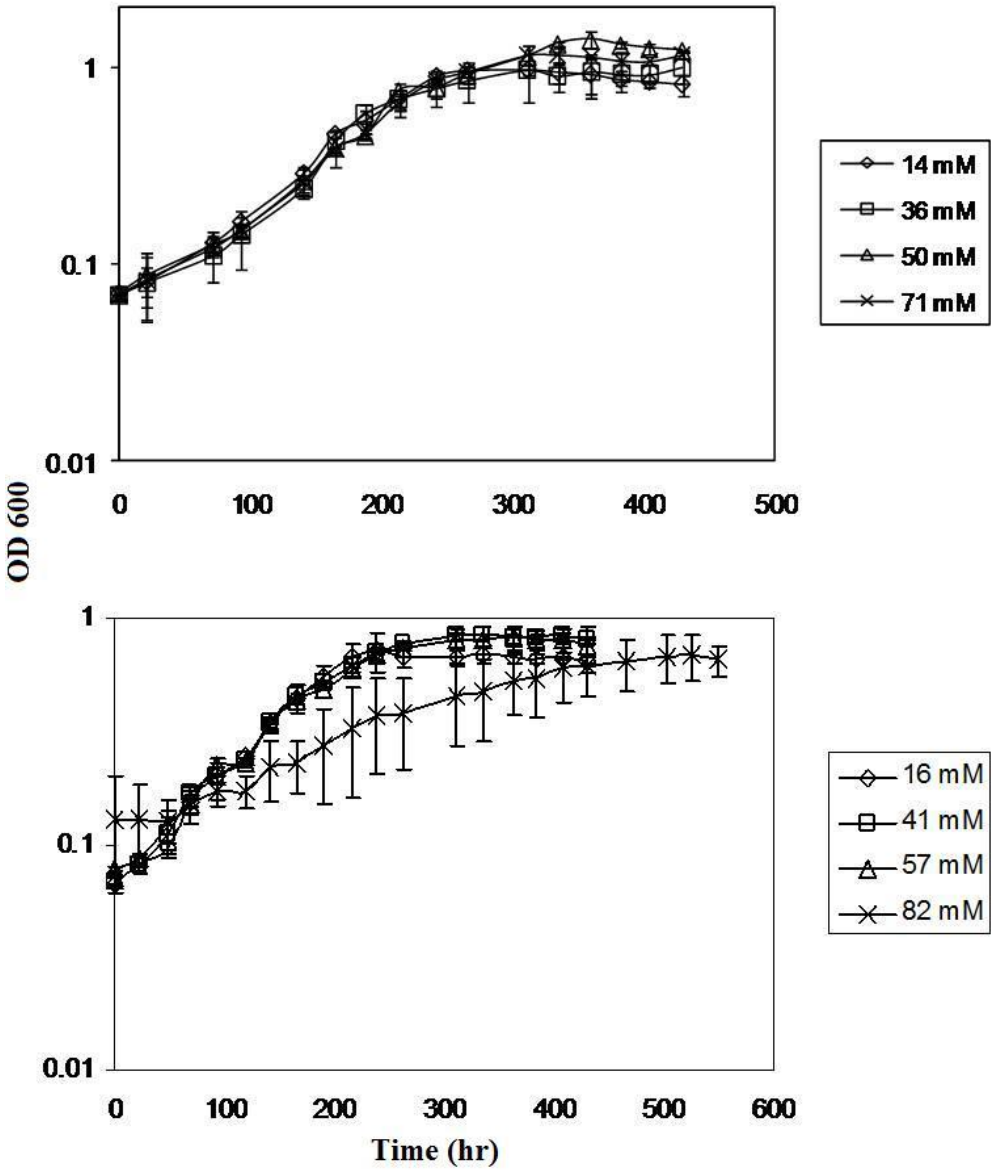


Figure 12. Pure Glycerol and Fractionated Glycerin Optimization for *D. alcoholvorans 6133* and *M. maripaludis*. (A) Growth on different concentrations of pure glycerol. (B) Growth on different concentrations of fractionated glycerin. Data points and experimental values are averages of two independent biological experiments (n=2).

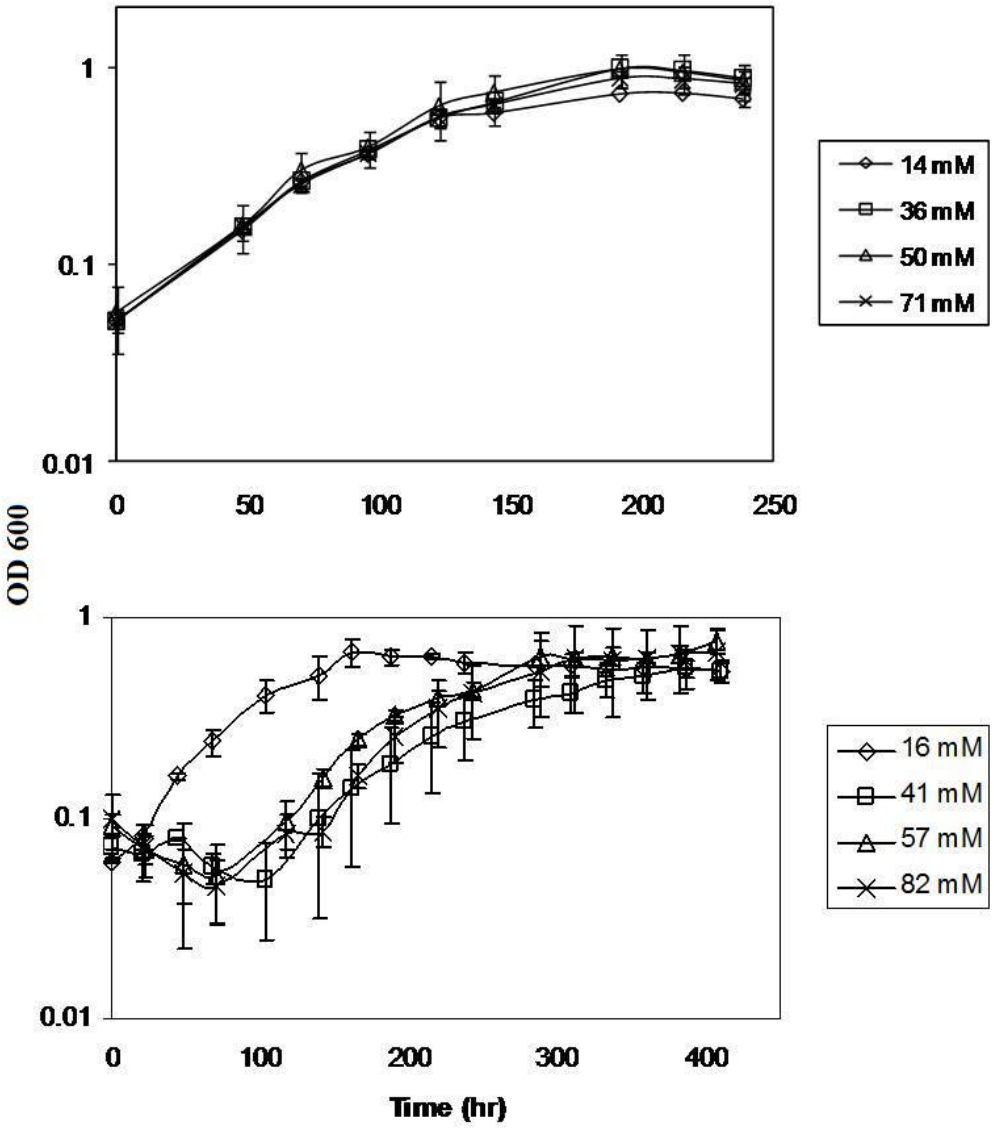


Figure 13. Pure Glycerol and Fractionated Glycerin Optimization for *D. vietnamensis & M. maripaludis*. (A) Growth on different concentrations of pure glycerol. (B) Growth on different concentrations of fractionated glycerin. Data points and experimental values are averages of two independent biological experiments (n=2).

Table 2. Methane Production from Cocultures Grown on Different Concentrations of Pure Glycerol.

Pure Glycerol	***D. alcoholvorans 6133 & M. maripaludis***		***D. vietnamensis & M. maripaludis***	
Initial Glycerol (mM)	**Final Glycerol (mM)**	**Methane (mM)**	**Final Glycerol (mM)**	**Methane (mM)**
14	0±0	11.8±2.38	0±0	10.6±0.78
36	19±10.1	11.2±1.53	0±0	19.8±1.24
50	18±6.3	17±6.04	11±0.08	12±4.53
71	30±5.0	16.5±1.28	14±5.27	12.9±4.30

Table 3. Methane Production from Cocultures Grown on Different Concentrations of Fractionated Glycerin.

Fractionated Glycerin	***D. alcoholvorans 6133 & M. maripaludis***		***D. vietnamensis & M. maripaludis***	
Initial Glycerol (mM)	**Final Glycerol (mM)**	**Methane (mM)**	**Final Glycerol (mM)**	**Methane (mM)**
16	0±0	13.4±1.90	0±0	8 ±9.98
41	4±3.96	13.5±9.03	0 ± 0	8±5.4
57	13±10.2	17.1±4.00	13±1.44	6 ±4.60
82	23±0.049	16.9±10.5	26±4.40	4±2.58

Coculture and Triculture Growth Experiments.

Coculture Growth on Pure Glycerol.

Growth rates and methane production varied for each coculture on pure glycerol, fractionated glycerin, and crude glycerin (Figures 16-21A-C). Cultures on pure glycerol displayed a small range of growth rates (0.003 h^{-1}). *D. vietnamensis* and *M. maripaludis* displayed the fastest growth rate with glycerol (0.01±0 h^{-1}) but one of the lower methane yields (0.4 mM CH_4/mM glycerol) (Figure 17A, Table 4). The highest methane yield (0.6 mM CH_4/mM glycerol) was produced by *D. vietnamensis* and *M. marisnigri* which also

experienced the slowest growth rate (0.007± 0.001 h^{-1}) and was the only culture that utilized almost 40 mM glycerol under the tested batch conditions (Figure 19, Table 4). *D. alcoholvorans* and *M. marisnigri* had the lowest methane yield (0.3 mM CH_4/mM glycerol) and the second fastest growth rate (0.009± 0.0003 h^{-1}) (Figure 18A, Table 4) while *D. alcoholvorans* and *M. maripaludis* had the second highest methane yield (0.5 mM CH_4/mM glycerol) and a slower growth rate (0.008±0 h^{-1}) (Figure 16A, Table 4). *D. alcoholvorans* and *M. maripaludis* utilized the least amount of glycerol at the end of growth.

Coculture Growth on Fractionated Glycerin.

Fractionated glycerin resulted in slower growth of each coculture compared to growth on pure glycerol (Figures 16-21B). *D. alcoholvorans 6133* and *M. marisnigri* displayed the slowest growth (0.004±0.001 h^{-1}) but produced the highest methane yield (0.5 mM/mM glycerol) which increased from growth on pure glycerol (Figure 18B, Table 4). *D. vietnamensis* and *M. maripaludis* was the only other culture that increased in methane production when grown on fractionated glycerin which also had the highest growth rate (0.007±0.004 h^{-1}) (Figure 17B, Table 4). Similar to results with glycerol, final acetate concentrations were in close agreement to the initial glycerol concentrations that each culture was provided.

Coculture Growth on Crude Glycerin.

With the exception of *D. alcoholvorans 6133* and *M. marisnigri*, every growth rate was lower on crude glycerin compared to growth on pure glycerol and fractionated glycerin (Figures 16-21C, Table 4). The growth rate for *D. alcoholvorans 6133* and *M. marisnigri* was the same as on fractionated glycerin; however, a lag of approximately 500 hours was experienced prior to exponential-phase growth (Figure 18C). *D. vietnamensis* and *M. marisnigri* also displayed a long lag-phase that lasted approximately 1,000 hours (Figure 19C), and the lag-phase was dependent upon the ratio of the inoculum size to initial glycerin level (data not shown). Both *D. alcoholvorans 6133* and *M. maripaludis* and *D. alcoholvorans 6133* and *M. marisnigri* methane yields increased, compared to growth on pure glycerol and fractionated glycerin, and were the highest on crude glycerin (0.6 CH_4 mM/mM glycerol).

Triculture Growth on Pure Glycerol.

Both tricultures grew at similar rates (0.008 ± 0.0004 h^{-1}) with similar methane yields (Figures 20 & 21A, Table 4). The triculture, *D. vietnamensis* and *M. maripaludis* and *M. marisnigri* did not have any glycerol that remained after growth while the other had approximately 5-5.5 mM of unconsumed glycerol.

Triculture Growth on Fractionated Glycerin**.**

Both tricultures grew slower on fractionated glycerin compared to pure glycerol Figures 20 & 21B, Table 4). *D. vietnamensis* and *M. maripaludis*, and *M. marisnigri* was the only culture to utilize all 57 mM fractionated glycerin. This triculture also displayed

the longest lag phase of 1,000 hours. The triculture, *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri,* was one of the fastest growing cultures on fractionated glycerin (0.006±0.001 h^{-1}). This triculture also produced the highest amount of methane (12.4±0.08 mM) on fractionated glycerin compared to pure glycerol and crude glycerin. Both tricultures produced similar amounts of methane concentration on fractionated glycerin.

Triculture Growth on Crude Glycerin**.**

Both tricultures grew slower on crude glycerin compared to fractionated glycerin Figures 20 & 21C, Table 4). *D. vietnamensis* and *M. maripaludis,* and *M. marisnigri* produced less methane on crude glycerin (6.5±3.0 mM) than on pure glycerol and crude glycerin. *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri* produced comparable concentrations of methane on crude glycerin as on pure glycerol (10.7±0.3 mM). Both tricultures had final acetate concentrations that were higher than initial crude glycerin concentrations.

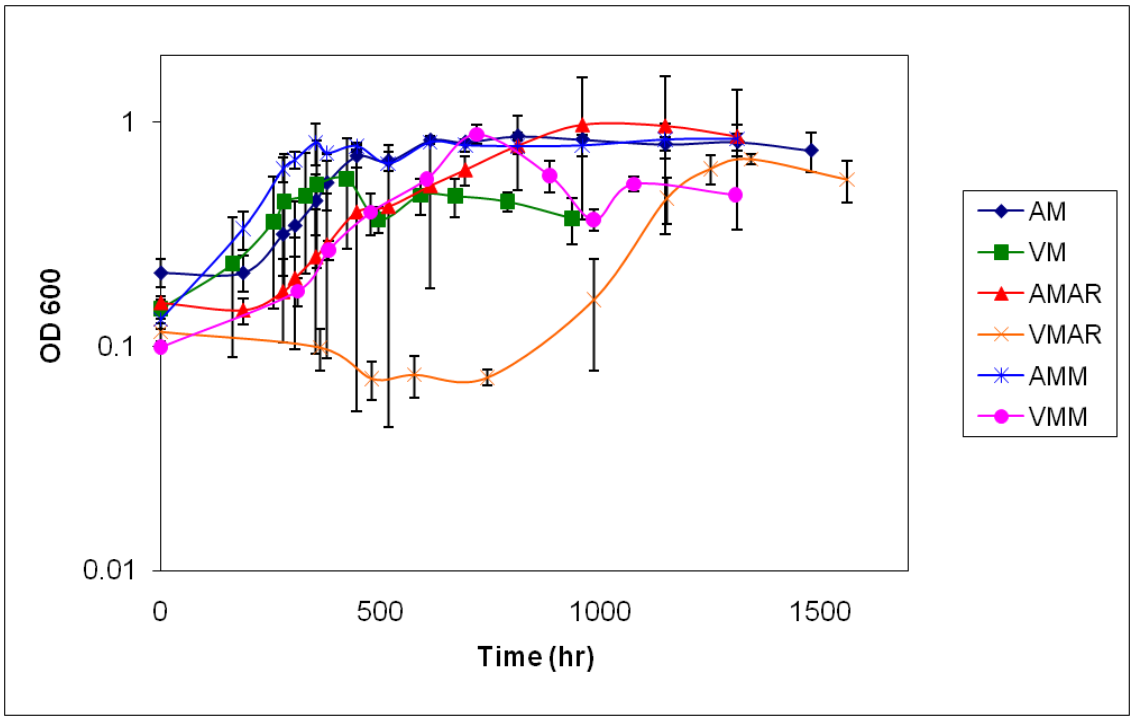


Figure 14. Growth Comparison on Fractionated Glycerin. AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri.* Data points are averages of two independent biological experiments (n=2).

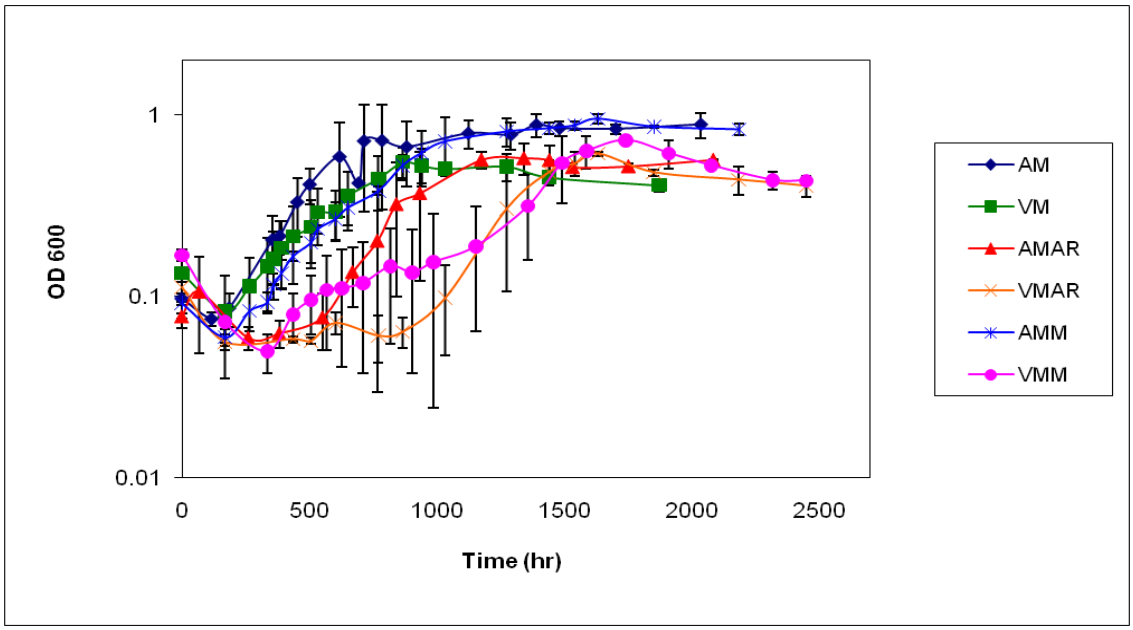


Figure 15. Growth Comparison on Crude Glycerin. AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*. Data points and are averages of two independent biological experiments (n=2).

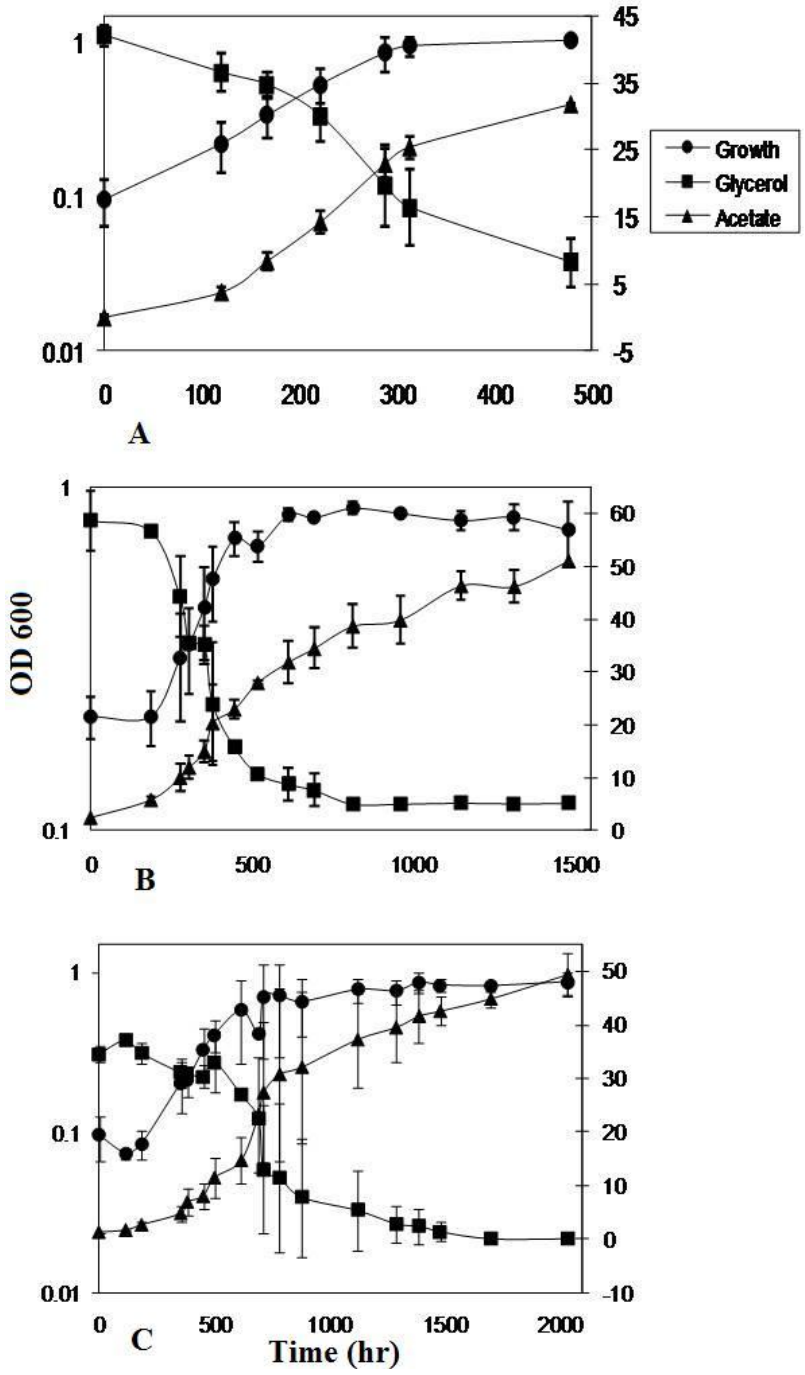


Figure 16. Growth of *D. alcoholvorans 6133* and *M. maripaludis* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

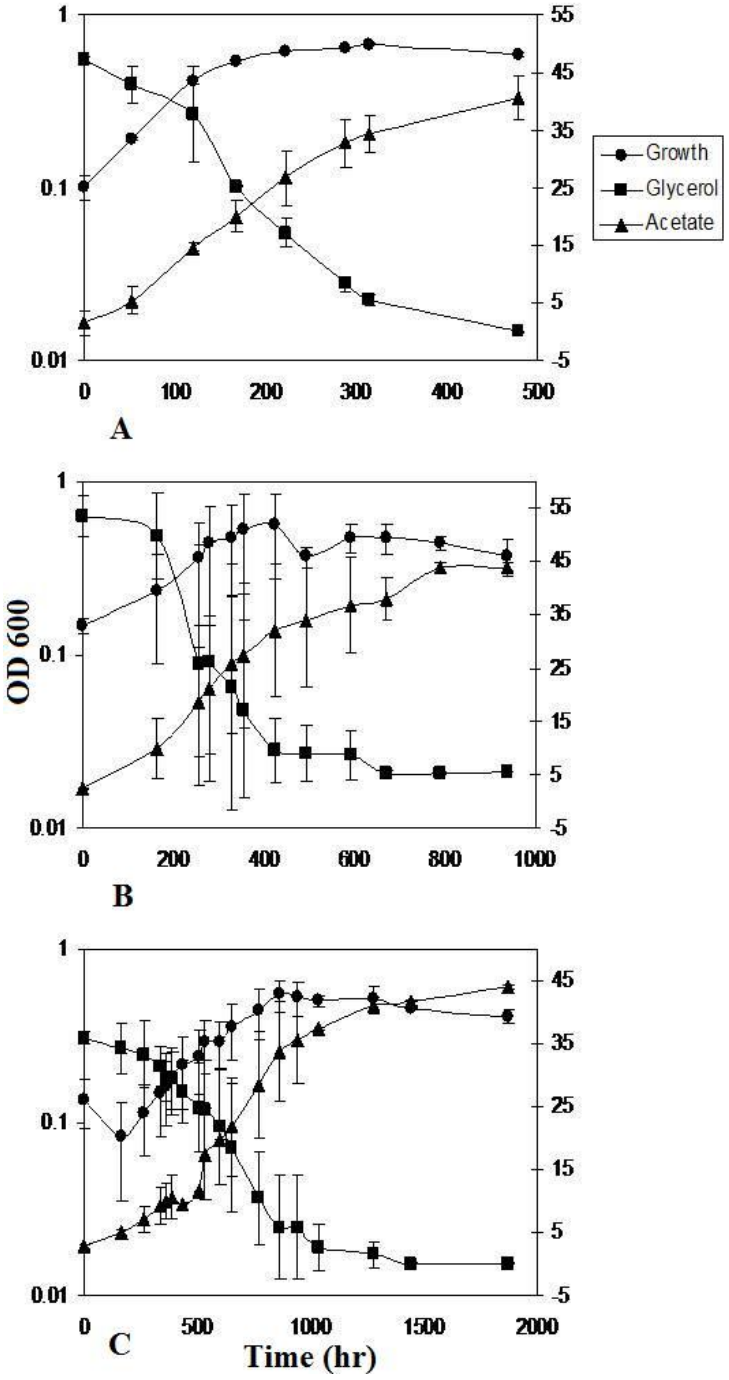


Figure 17. Growth of *D. vietnamensis* and *M. maripaludis* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

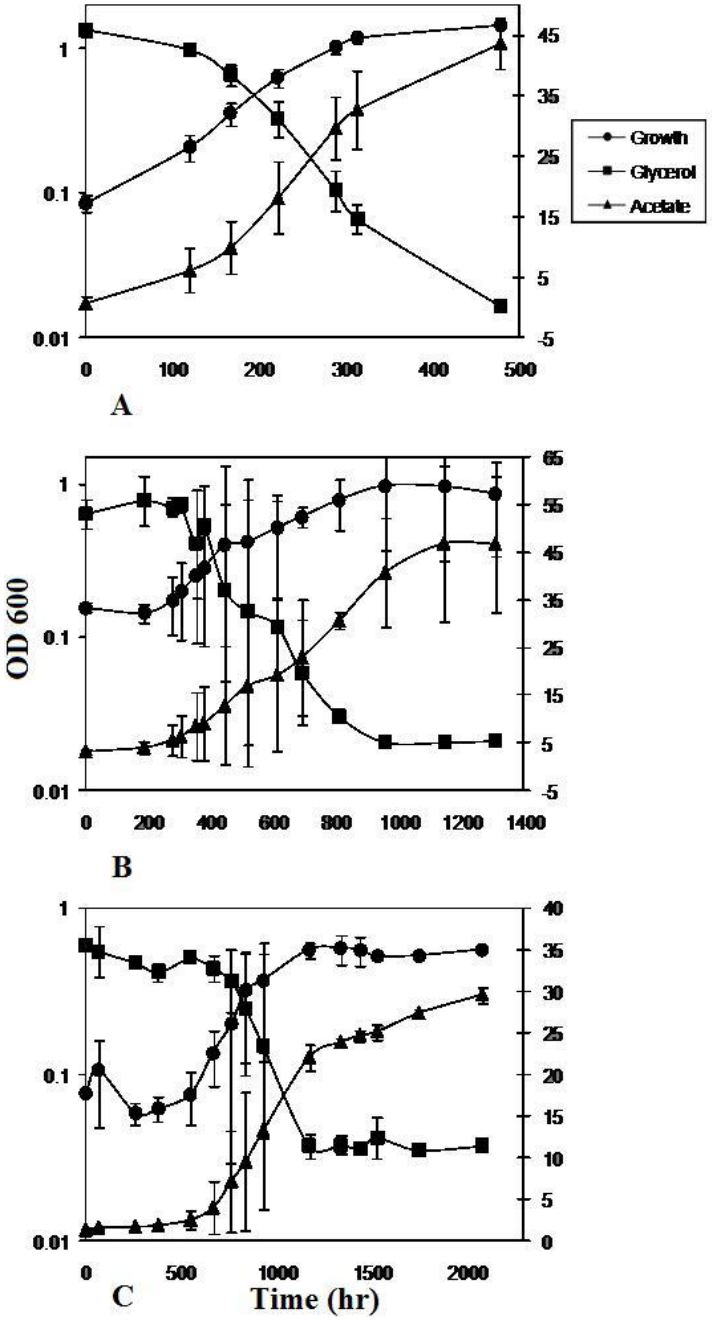


Figure 18. Growth of *D. alcoholvorans 6133* and *M. marisnigri* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

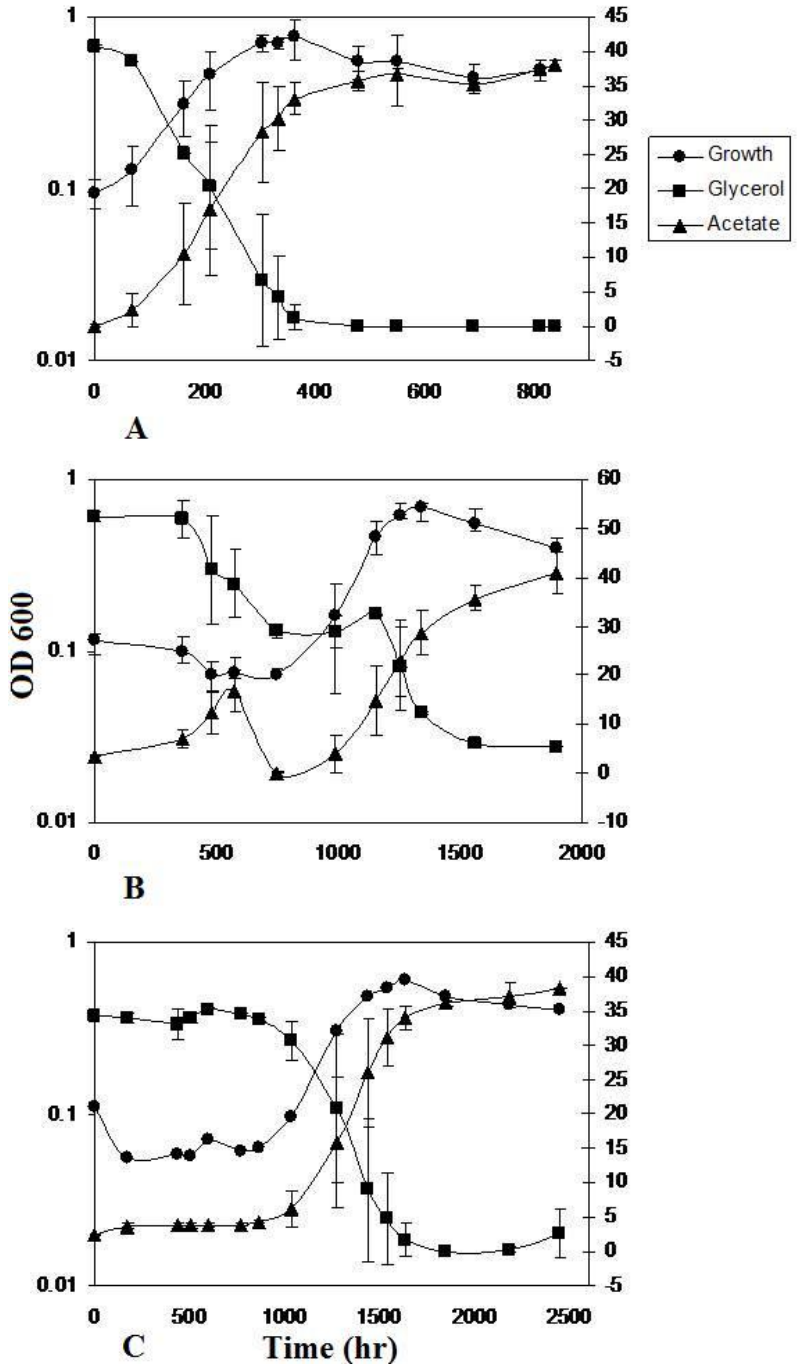


Figure 19. Growth on *D. vietnamensis* and *M. marisnigri* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

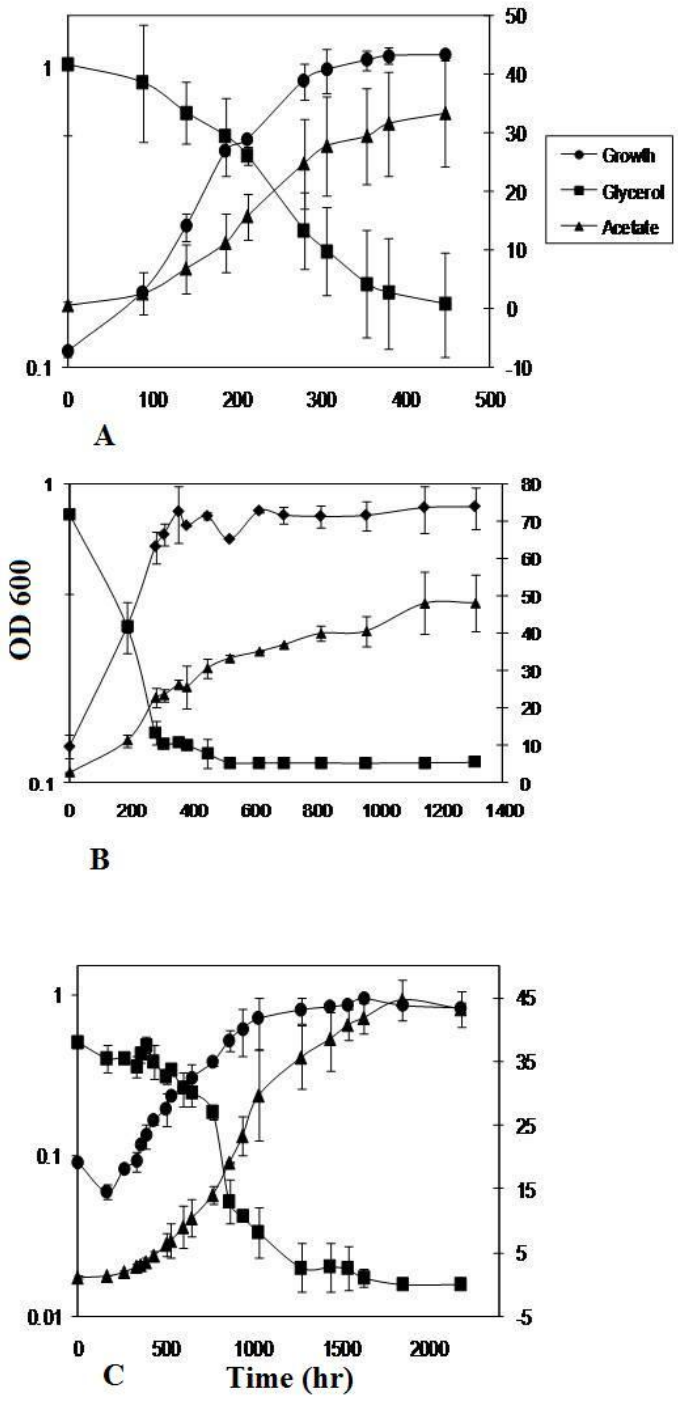


Figure 20. Growth on *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

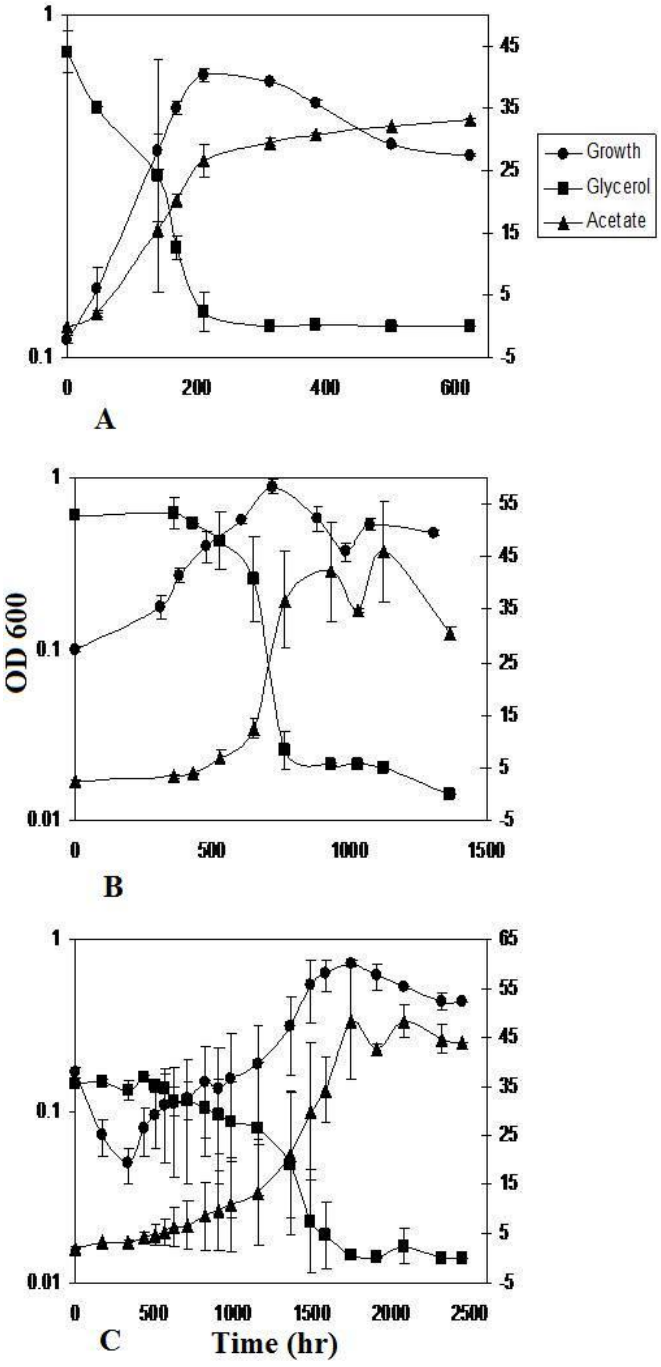


Figure 21. Growth of *D. vietnamensis* and *M. maripaludis* and *M. marisnigri* on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. (A) Growth on 36 mM pure glycerol. (B) Growth on 57 mM fractionated glycerin (based on glycerol concentration). (C) Growth on 37 mM crude glycerin (based on glycerol concentration). Data points are averages of two independent biological experiments (n=2).

Table 4. Methane Production from Growth on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin.

	Methane (mM)		
Co/Triculture	**Pure Glycerol**	**Fractionated Glycerin**	**Crude Glycerin**
D. alcoholvorans 6133 & M. maripaludis	11.5±5.4	11.7±0.431	12.9±3.81
D. vietnamensis & M. maripaludis	11.9±5.41	15.2±0.359	9.6±4.57
D. alcoholvorans 6133 & M. marisnigri	9.6±6.60	15.5±6.26	8.9±1.19
D. vietnamensis & M. marisnigri	15.3±0	11.3±0.063	7.2±3.35
D. alcoholvorans 6133 & M. maripaludis & M. marisnigri	10.3±4.68	12.4±0.078	10.7±0.34
D. vietnamensis & M. maripaludis & M. marisnigri	12.5±0.99	13.6±2.12	6.5±2.99

Free Fatty Acids.

Free fatty acid concentrations were also measured at the beginning and end of growth on fractionized and crude glycerin (Figure 22). For each culture, the free fatty acid concentration was only slightly increased (by approximately 0.02-0.03 mM) at the end of growth compared to initial levels.

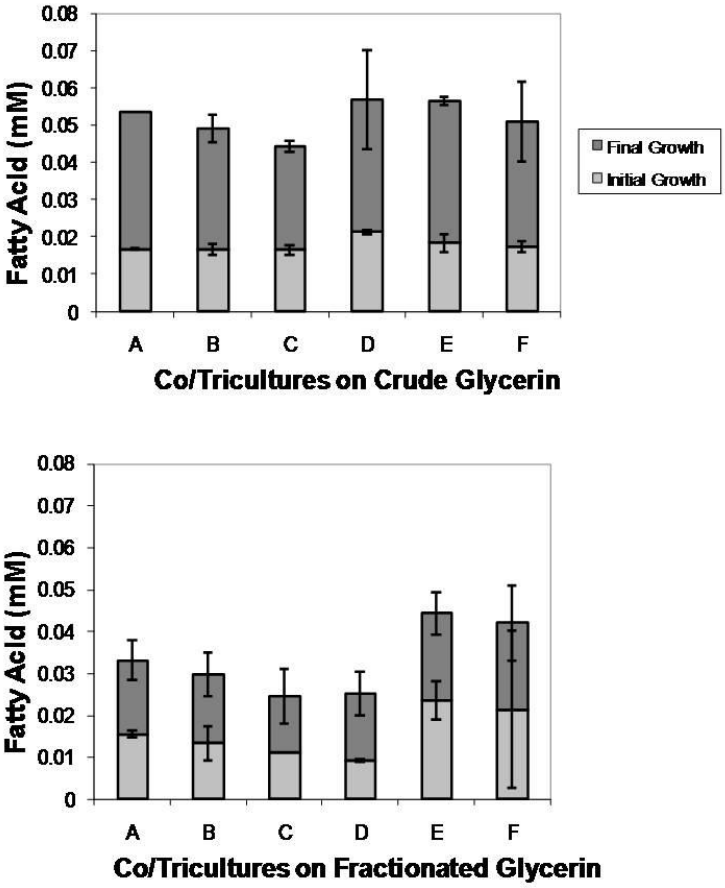


Figure 22. Free Fatty Acids. (A) *D. alcoholvorans 6133* and *M. maripaludis.* (B) *D. vietnamensis* and *M. maripaludis.* (C) *D. alcoholvorans 6133* and *M. marisnigri.* (D) *D. vietnamensis* and *M. marisnigri.* (E) *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri.* (F) *D. vietnamensis* and *M. maripaludis* and *M. marisnigri.* Free fatty acid concentrations were measured at the beginning and end of growth. Experimental values are averages of two independent biological experiments (n=2).

Methanosarcina acetivorans Growth Experiments.

M. acetivorans Growth on Methanol and Crude Glycerin with Various Headspace Concentrations.

M. acetivorans produced 79±13.01 mM CH_4 and reached an optical density of 0.98 when grown under 20% CO_2: 80% N_2 headspace on 150 mM methanol (Figure

23A). When grown on crude glycerin (37 mM) under 20% CO_2: 80% N_2 headspace, *M. acetivorans* utilized the methanol fraction (33 mM) as the carbon and energy source and produced 18.2±4.42 mM CH_4 reaching an optical density of approximately 0.2 (Figure 23B). *M. acetivorans* did not grow with sodium acetate compared to methanol in the coculture media without considerable lag-phases (>450 h). Cultures of *M. acetivorans* were grown on different CO_2 and N_2 headspaces in order to test if higher CO_2 concentrations in the headspace affected growth with acetate, and results indicated that methanol was the preferred substrate in the tested growth medium regardless of CO_2 levels (Figure 24A&B). When the *M. acetivorans* cultures grown on sodium acetate were transferred to the same media and given sodium acetate, the lag-phase decreased to 18.5 hours and 86±2.7 mM CH_4 was produced.

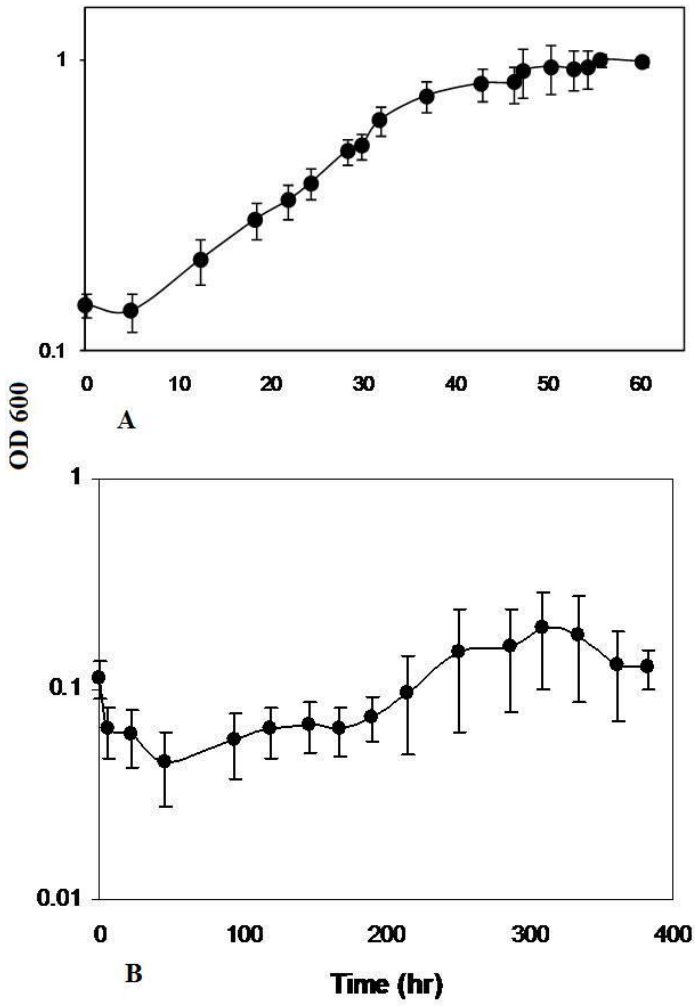


Figure 23. *M. acetivorans* Growth on Methanol and Crude Glycerin. (A) Growth on 150 mM methanol. (B) Cultures grown on 33 mM methanol fraction of 37 mM crude glycerin. Both cultures grown under 20% CO_2 and 80% N_2 headspace. Data points are averages of two independent biological experiments (n=2).

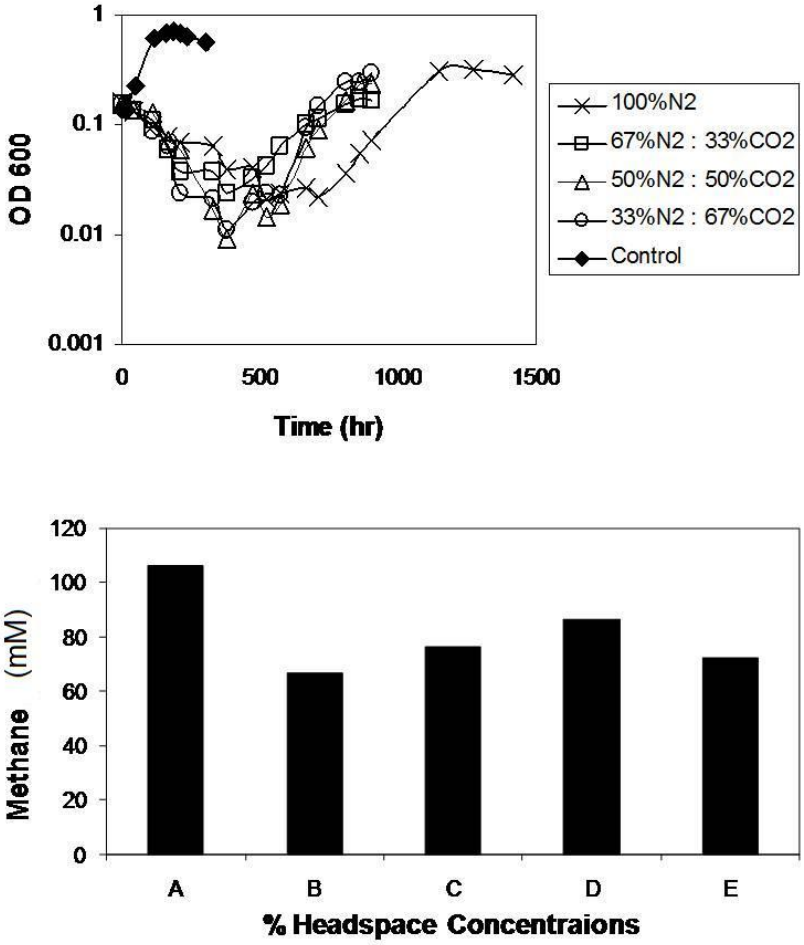


Figure 24. Growth of *M. acetivorans* Under Different Headspace Concentrations and Methane Production. (A) ♦ (control) grown on 150 mM methanol under 100% N_2 headspace. (B) x Growth on 120 mM sodium acetate under 100% N_2 headspace. (C) □ Growth on 120 mM sodium acetate under 67% N_2 and 33% CO_2. (D) Δ Growth on 120 mM sodium acetate under 50% N_2 and CO_2 headspace. (E) ○ Growth on 120 mM sodium acetate under 33% N_2 and 67% CO_2. Data points are from one independent biological experiment (n=1).

SRB and Hydrogenotrophic Methanogen Cocultures with *M. acetivorans.*

Cultures with *M. acetivorans* were established in two ways: either cocultures were combined with half the concentration of *M. acetivorans* (Triculture A) or equal concentrations of coculture and *M. acetivorans* were combined (Triculture B) (Figure

25A-D, Table 5). For example, Triculture A was established for *D. vietnamensis*, *M. maripaludis*, and *M. acetivorans* by inoculating a serum bottle with a late-exponential phase coculture of *D. vietnamensis* and *M. maripaludis* to an optical density of approximately 0.07-1.0. Then, a culture of *M. acetivorans* grown to late-exponential phase was inoculated into the same serum bottle with an initial optical density of 0.04-0.05. Triculture B was established the same way, but *M. acetivorans* was inoculated with the same starting optical density as the coculture (0.07-1.0).

For the most part, methane concentrations were much higher with the addition of *M. acetivorans*. *M. acetivorans* displayed the capability of utilizing the acetate by-product of SRB glycerol oxidation (data not shown). For B Tricultures, methane concentrations increased considerably when *M. acetivorans* was added to the cocultures: *D. vietnamensis* and *M. maripaludis* (from 9.5 to 75.2 mM CH_4), *D. alcoholvorans 6133* and *M. maripaludis* (from 12.9 to 58.3 mM CH_4), and *D. alcoholvorans 6133* and *M. marisnigri* (from 8.9 to 69.9 mM CH_4) (Figure 25A-C, Table 5). The only culture that decreased in methane production with the addition of *M. acetivorans* was the *D. vietnamensis* and *M. marisnigri* which lowered to 3.4 from 7.2 mM CH_4 (Figure 25D, Table 5).

Methane increased in all A Tricultures compared to coculture growth on crude glycerin without *M. acetivorans*. Aside from *D. vietnamensis* and *M. marisnigri*, all Triculture A methane values were lower than Triculture B values. The methane values from A Tricultures were: 30.5 mM CH_4 from *D. vietnamensis* and *M. maripaludis*, 47.1 mM CH_4 from *D. alcoholvorans 6133* and *M. maripaludis*, 50.32 mM CH_4 from *D.*

alcoholvorans 6133 and *M. marisnigri*, and 21.1 mM CH_4 from *D. vietnamensis* and *M. marisnigri*.

D. vietnamensis and *M. maripaludis* Triculture A and B grew at similar rates but Triculture B reached the highest optical density. Both Triculture A and B grew faster and to a higher optical density than *D. vietnamensis* and *M. maripaludis* grown as cocultures. The triculture B with *D. alcoholvorans 6133* and *M. maripaludis* grew the fastest. Triculture A and coculture growth grew at the same rate and eventually reached the same optical density as Triculture B. *D. alcoholvorans 6133* and *M. marisnigri* was the only culture to grow fastesr as Triculture A, however Triculture B reached the highest optical density. Both Triculture A and B grew faster and to a higher optical density than *D. alcoholvorans 6133* and *M. marisnigri* grown as a coculture. Triculture B with *D. vietnamensis* and *M. marisnigri* reached the highest optical density with the shortest lag period (217 hours). Both Triculture A and coculture growth had long lag times (550 and 865 hours, respectively). Triculture A grew faster than *D. vietnamensis* and *M. marisnigri* as a coculture but the coculture grew to a higher optical density (Figure 25A-D, Table 5).

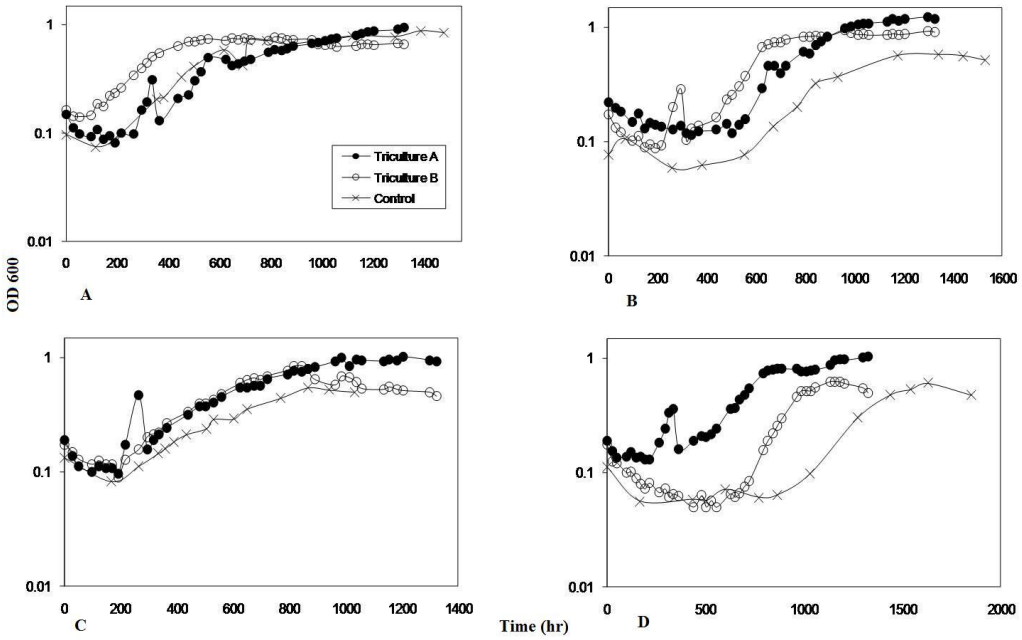


Figure 25. Tricultures: SRB and Hydrogenotrophic Methanogen Cocultures Combined with *M. acetivorans.* (A) *D. alcoholvorans 6133* and *M. maripaludis* (B) *D. alcoholvorans 6133* and *M. marisnigri* (C) *D. vietnamensis* and *M. maripaludis* (D) *D. vietnamensis* and *M. marisnigri*. Cultures were grown on 37 mM crude glycerin (33 mM methanol). Starting optical densities for Triculture A were approximately 0.04-0.05 and 0.07-0.1 for *M. acetivorans* and cocultures, respectively. For Triculture B, starting optical densities were approximately 0.08-0.1 for both *M. acetivorans* and cocultures. Controls are cocultures grown in the same conditions without *M. acetivorans*. Data points are from one independent biological experiment (n=1).

Table 5. Methane Production of *M. acetivorans* with Cocultures. Methane measured when cultures reached late-stationary phase.

***M. acetivorans* with**	**Cocultures Only (mM CH_4)**	**Triculture A (mM CH_4)**	**Triculture B (mM CH_4)**
D. alcoholvorans 6133 & M. maripaludis	12.5	47.1	58.3
D. vietnamensis & M. maripaludis	9.5	30.5	75.2
D. alcoholvorans 6133 & M. marisnigri	8.9	50.3	69.9
D. vietnamensis & M. marisnigri	7.2	21.1	3.4

Cocultures Grown on *M. acetivorans* Supernatant.

D. alcoholvorans 6133 and *M. marisnigri* and *D. vietnamensis* and *M. marisnigri* grew much faster and produced more methane (16.7 and 11.3 mM CH_4, respectively) on the supernatant of *M. acetivorans* growing on the methanol fraction (33 mM) of crude glycerin (37 mM) (Figure 23A) than when directly grown as cocultures on crude glycerin (Figures 18&19D). *D. alcoholvorans 6133* and *M. marisnigri* did not experience a lag period, reached a higher optical density, and the *D. vietnamensis* and *M. marisnigri* lag-phase was approximately seven times shorter (124 hours) when grown on *M. acetivorans* supernatant. Both cocultures with *M. maripaludis* produced a lower concentration of methane (7.2 and 6.3 mM CH_4 from *D. vietnamensis* or *D. alcoholvorans 6133,* respectively). Both, *D. vietnamensis* and *M. maripaludis* and *D. alcoholvorans 6133* and *M. maripaludis,* grew to a much lower optical density when grown on the supernatant of *M. acetivorans* (Figure 26A-D, Table 6) (0.177 and 0.291, respectively).

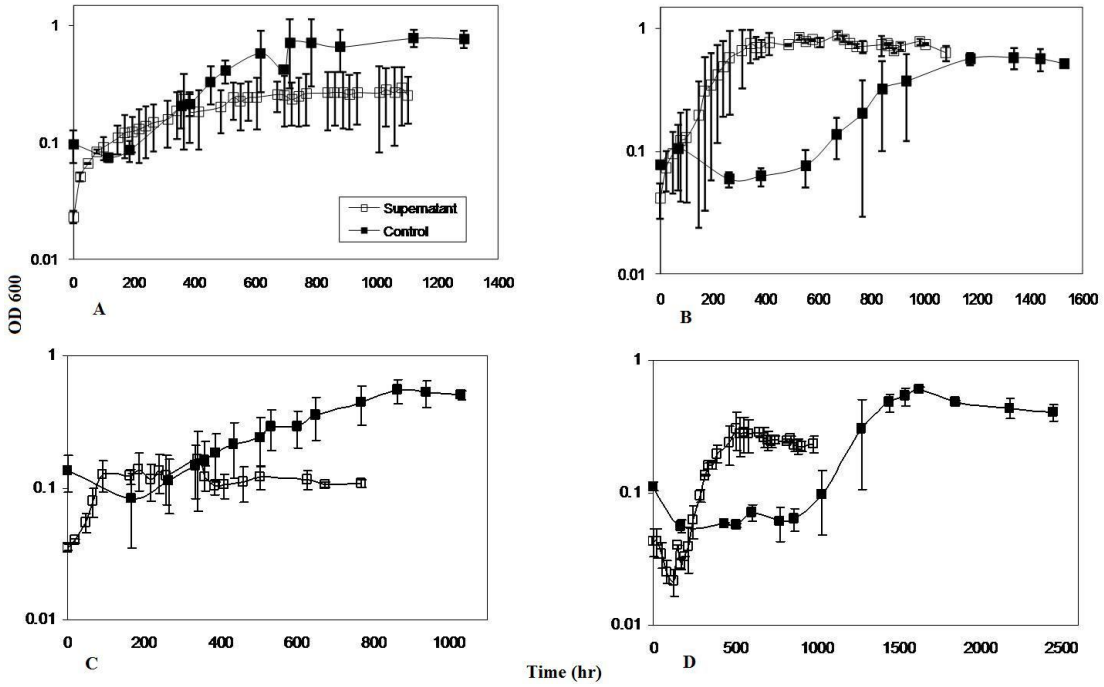


Figure 26. Cocultures Growth on *M. acetivorans* Supernatant. (A) *D. alcoholvorans 6133* and *M. maripaludis* (B) *D. alcoholvorans 6133* and *M. marisnigri* (C). *D. vietnamensis* and *M. maripaludis* (D) D. *vietnamensis* and *M. marisnigri*. *M. acetivorans* was grown on 33 mM methanol from 37 mM crude glycerin (Figure 23B). After methane was measured, cells were spun down and filtered out. Cocultures were inoculated into the supernatant and utilized the crude glycerin as a carbon and energy source. Controls are cocultures grown on 37 mM crude glycerin. Data points are averages of two independent biological experiments (n=2).

Table 6. Methane Concentrations from Supernatant Growth.

Cocultures	**Control (mM CH_4)**	**Growth on Supernatant (mM CH_4)**
D. alcoholvorans 6133 & M. maripaludis	12.9±3.81	6.3±2.90
D. vietnamensis & M. maripaludis	9.5±4.57	7.2±0.025
D. alcoholvorans 6133 & M. marisnigri	8.9±1.19	16.7±1.86
D. vietnamensis & M. marisnigri	7.2±3.35	11.3±0.007

DISCUSSION AND CONCLUSIONS

Entire Consortia Physiology

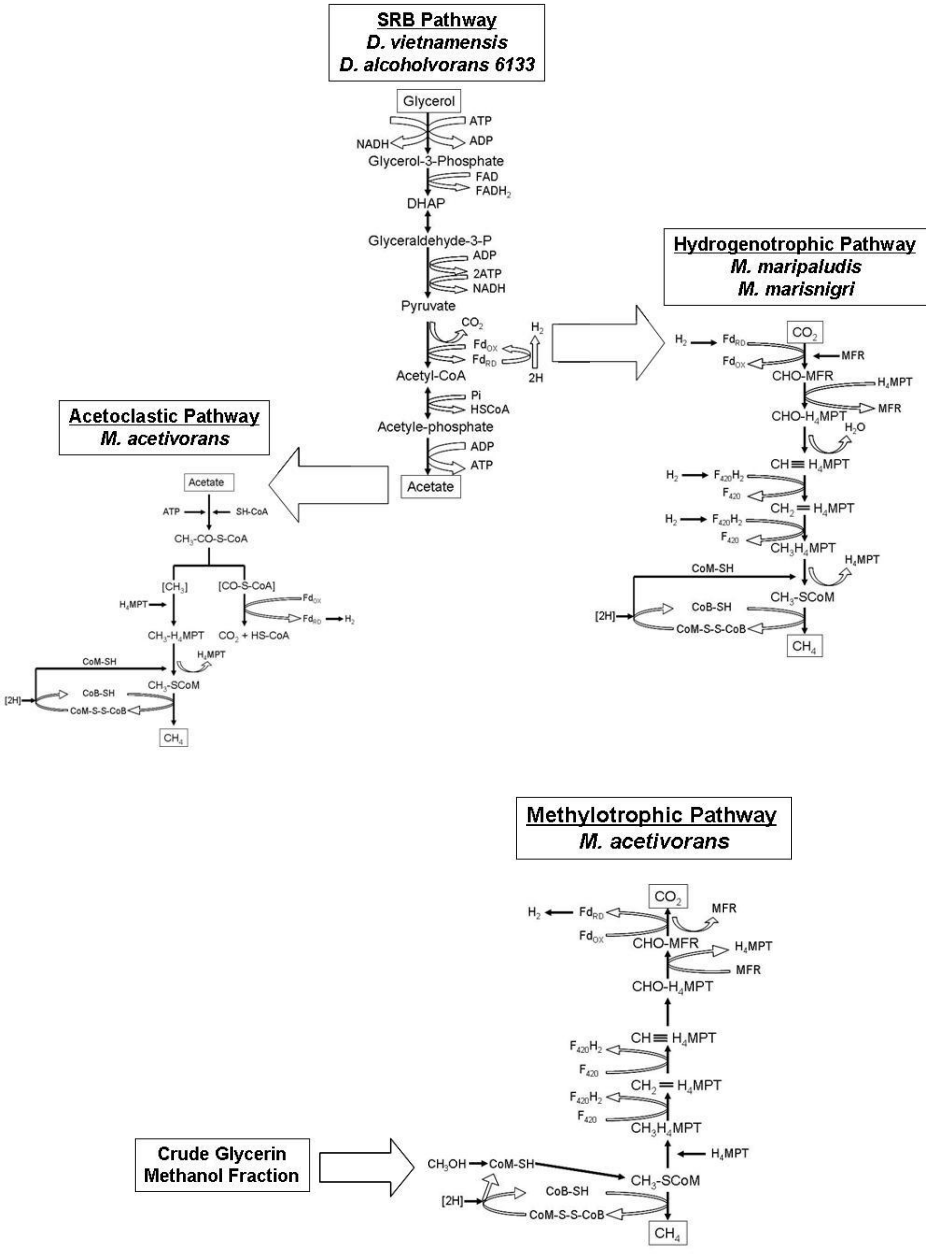


Figure 27. Complete Pathway of Co/Tricultures on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin.

Co/tricultures on Pure Glycerol, Fractionated Glycerin, and Crude Glycerin.

To gain deeper insight on the physiology of co/tricultures on pure glycerol, fractionated glycerin, and crude glycerin, growth rates, methane yields, and percent electron equivalents were calculated (Tables 7-9). Methane yields (Y_{CH4}) were determined by calculating the ratio of methane produced (mM CH_4) to the glycerol consumed (mM glycerol) after incomplete oxidation to acetate by the chosen SRBs (final mM CH_4)/(Δ mM glycerol). Percent electron equivalent determines the percentage of electrons that were transferred from glycerol to form methane. Glycerol has 14 electrons while methane has 8. When glycerol is partially oxidized to acetate, 8 electrons remain on the acetate molecule. This leaves 6 electrons that each have the potential of going into producing methane. Electron equivalent can then be calculated by taking the ratio of the total number of electrons in methane produced to the total number of electrons oxidized from glycerol that did not end up in acetate; (total mM CH_4 x 8 electrons)/(Δ mM glycerol x 6 electrons). Methane yield and percent electron equivalent are directly proportional.

Pure Glycerol.

Table 7. Co/tricultures on Pure Glycerol.

Co/Tricultures	Growth Rate (h^{-1})	Y_{CH4} (mM/mM)	Percent Electron Equivalent
D. alcoholvorans 6133 & M. maripaludis	0.008±0	0.507±0.070	67.6%
D. vietnamensis & M. maripaludis	0.010±0	0.377±0.037	50.3%
D. alcoholvorans 6133 & M. marisnigri	0.009±0.0003	0.309±0.005	41.2%
D. vietnamensis & M. marisnigri	0.007±0.0007	0.565	75.4%
D. alcoholvorans 6133 & M. maripaludis & M. marisnigri	0.008±0.0004	0.379±0.852	50.5%
D. vietnamensis & M. maripaludis & M. marisnigri	0.008±0.0004	0.427±0.091	56.9%

In order to understand trends between growth rates and methane yields (percent electron equivalents can be assumed to follow the same trend as methane yields since they are directly proportional), co/tricultures were ranked 1 to 6, with 1 being the lowest growth rate or methane yield and 6 being the highest of both. Co/tricultures were plotted based on their 2 rankings (Figure 28). In general, as growth rate increased on pure glycerol, methane yield decreased. The fastest growing cultures were *D. alcoholvorans 6133* and *M. marisnigri* and *D. vietnamensis* and *M. maripaludis* which were also the lowest methane producing cultures (Figures 17&18, Table 4). On the other hand, *D. vietnamensis* and *M. marisnigri* and *D. alcoholvorans 6133* and *M. maripaludis* grew the

slowest and produced the highest amount of methane (Figures 28, 16 & 19, Tables 4 & 7). Relative to the other cultures, both tricultures had moderate growth rates and methane yields. This is interesting because it suggests that the presence of both methanogens creates some type of middle ground for growth rate and methane yield. A trend was not observed between microorganism combinations, growth rates, or methane yields, although it appeared that with glycerol *D. vietnamensis* did better with *M. marisnigri* while *D. alcoholvorans* did better with *M. maripaludis*.

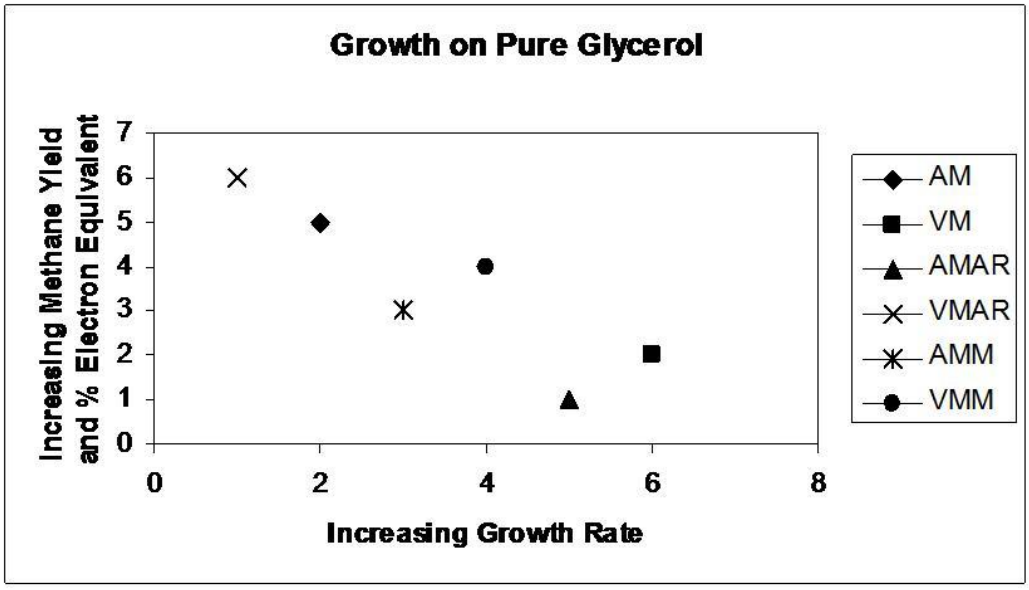


Figure 28. Growth Correlation on Pure Glycerol. Higher numbers represent faster growth and higher methane yields and higher percent electron equivalents. AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*.

Fractionated Glycerin.

Table 8. Co/tricultures on Fractionated Glycerin.

Co/Tricultures	Growth Rate (h^{-1})	Y_{CH4} (mM/mM)	Percent Electron Equivalent
D. alcoholvorans 6133 & M. maripaludis	0.005±0.0003	0.328±0.0323	43.7%
D. vietnamensis & M. maripaludis	0.007±0.0039	0.474±0.0724	63.2%
D. alcoholvorans 6133 & M. marisnigri	0.004±0.0008	0.486±0.3090	64.8%
D. vietnamensis & M. marisnigri	0.006 ± 0.0025	0.360±0.0073	48.0%
D. alcoholvorans 6133 & M. maripaludis & M. marisnigri	0.006±0.0010	0.280±0.1253	37.4%
D. vietnamensis & M. maripaludis & M. marisnigri	0.005±0.0011	0.385±0.0003	51.4%

With the exception of *D. vietnamensis* and *M. maripaludis*, cultures followed a similar trend where faster growth resulted in lower methane yields with fractionated glycerin (Figure 29, Table 8). As cocultures, both SRB grew faster when paired with *M. maripaludis* than with *M. marisnigri*, and this also corresponded to slower glycerol consumption rates with *M. marisnigri*. *D. vietnamensis* and *M. maripaludis* produced the second highest methane yield with the highest growth rate. These results suggested that *M. marisnigri* might be more susceptible to contaminants in the fractionated glycerin. Interestingly, tricultures with *D. alcoholvorans 6133* produced less methane than cocultures with *D. alcoholvorans 6133*. The methanogens produced more methane when

grown separately in this case. Similar to growth on pure glycerol, trends were not observed between the type of SRB and methanogen coupling, growth rate, or methane yield on fractionated glycerin.

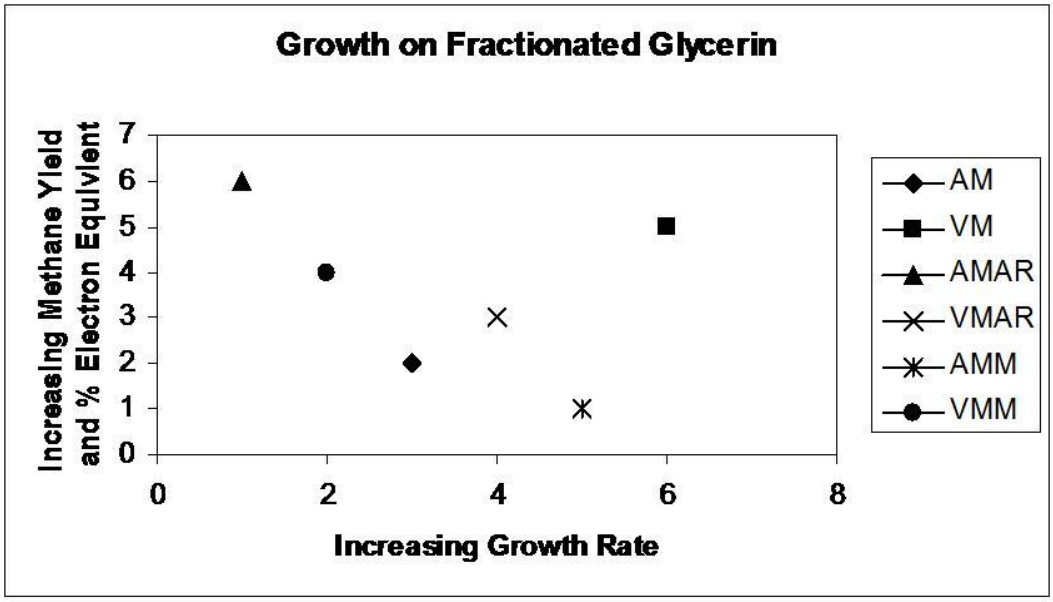


Figure 29. Growth Correlation on Fractionated Glycerin. Higher numbers represent faster growth and higher methane yields and higher percent electron equivalents. AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*.

Crude Glycerin.

Table 9. Co/tricultures on Crude Glycerin.

Co/Tricultures	Growth Rate (h^{-1})	Y_{CH4} (mM/mM)	Percent Electron Equivalent
D. alcoholvorans 6133 & M. maripaludis	0.003±0.0001	0.559±0.1877	74.5%
D. vietnamensis & M. maripaludis	0.003±0.0002	0.401±0.0599	53.3%
D. alcoholvorans 6133 & M. marisnigri	0.004±0.0008	0.552±0.8444	73.6%
D. vietnamensis & M. marisnigri	0.003±0.0005	0.343±0.1029	45.7%
D. alcoholvorans 6133 & M. maripaludis & M. marisnigri	0.002± 0.0006	0.280±0.0274	37.4%
D. vietnamensis & M. maripaludis & M. marisnigri	0.004±0.0027	0.273±0.0558	36.4%

With the exception of *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*, cultures appear to follow the opposite trend when grown on crude glycerin; methane yield increases with growth rate (Figure 30, Table 9). Both tricultures had the lowest methane yields, perhaps competition exists between the two hydrogenotrophic methanogens under these conditions. Again with crude glycerin, trends were not seen between type of SRB and methanogen coupling, growth rate, and methane yield.

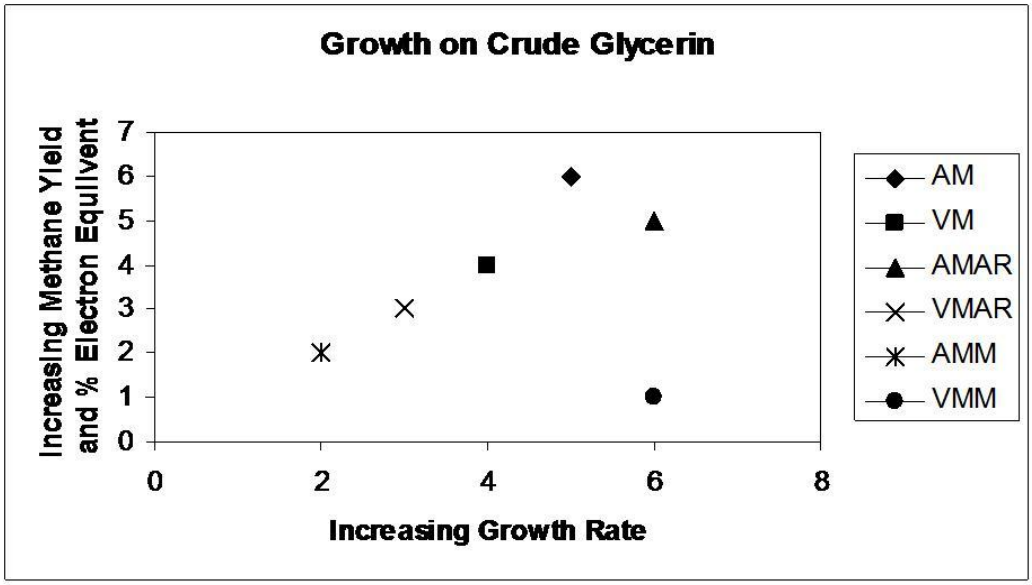


Figure 30. Growth Correlation on Crude Glycerin. Higher numbers represent faster growth and higher methane yields and higher percent electron equivalents. AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*.

Correlations Between Pure Glycerol, Fractionated Glycerin, and Crude Glycerin.

Figure 31 is a graph plotting every culture's growth rate and methane yield. The only trend that existed was between growth rate and carbon source. Cultures grown on crude glycerin displayed the lowest growth rates, while the growth rates for glycerol-grown cultures had the highest growth rates. There is not an overall trend between growth rate and methane yield. The trend only seems to exist within each individual carbon source. Methane yield was lowest when the growth rate was greater than 0.004 h^{-1} and slower than 0.007 h^{-1}. Again, there was no trend between culture combination, growth rate, and methane yield. Different culture combinations had improved methane conversions and increased growth rates dependent upon which carbon and energy source

was used. Overall, glycerol-grown cultures had the highest combination for both growth rate and methane yield. Cultures grown with crude glycerin, namely *D. alcoholvorans* with *M. maripaludis* or *M. marisnigri* had comparable methane yields to glycerol-grown cultures but the growth rates were approximately 2-fold lower.

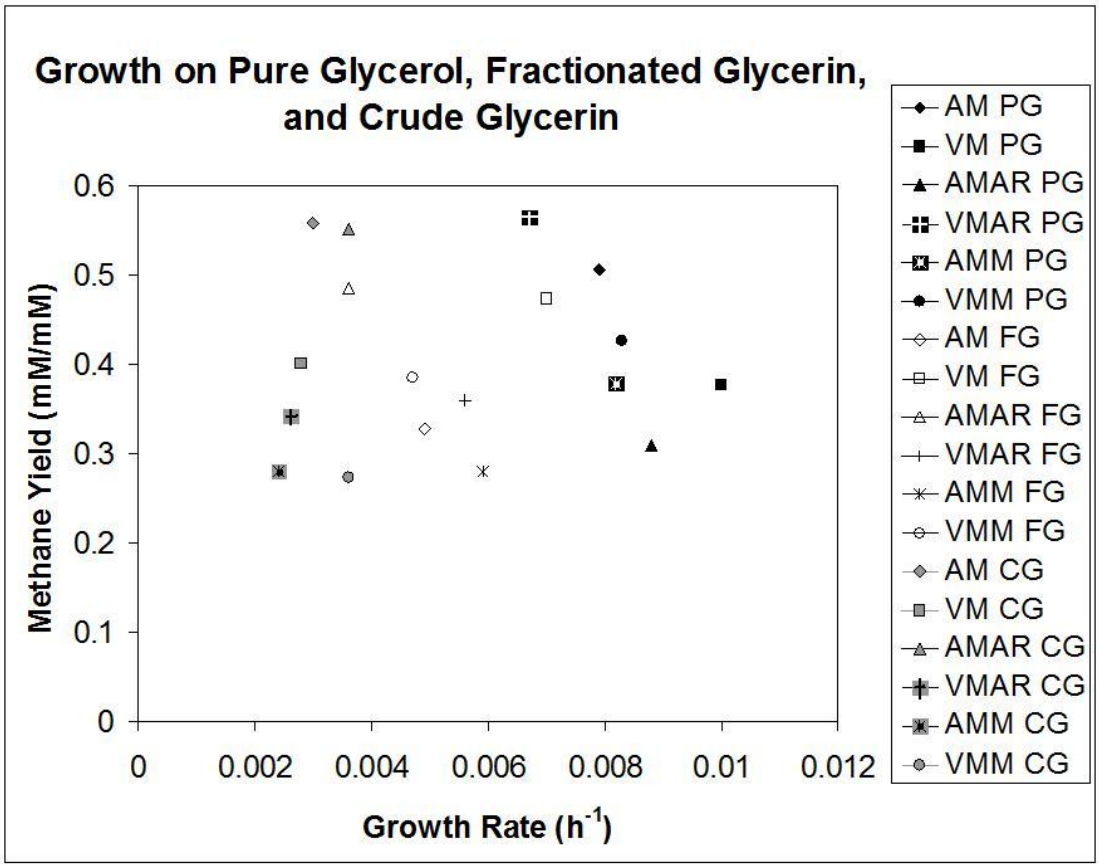


Figure 31. Correlations Between Pure Glycerol, Fractionated Glycerin, and Crude Glycerin. PG: pure glycerol, FG: fractionated glycerin, CG: crude glycerin, AM: *D. alcoholvorans 6133* and *M. maripaludis*, VM: *D. vietnamensis* and *M. maripaludis*, AMAR: *D. alcoholvorans 6133* and *M. marisnigri*, VMAR: *D. vietnamensis* and *M. marisnigri*, AMM: *D. alcoholvorans 6133* and *M. maripaludis* and *M. marisnigri*, VMM: *D. vietnamensis* and *M. maripaludis* and *M. marisnigri*.

M. acetivorans Experiments.

An interesting peak was seen at approximately 300 hours when *M. acetivorans* was grown with each of the cocultures on 37 mM crude glycerin. The peak occurred at about the same time that *M. acetivorans* reached stationary phase when grown as a monoculture on the methanol fraction (33 mM) of 37 mM crude glycerin. Therefore, the peak most likely represents *M. acetivorans* utilizing the methanol fraction from crude glycerin as the coculture is just entering exponential phase. Once the methanol was used up, *M. acetivorans* probably slowly started to consume acetate being produced by the SRB, which could account for the higher methane production. Although, *M. acetivorans* did not reach a high optical density when grown on acetate, it still produced a significant amount of methane.

With the exception of *D. vietnamensis* and *M. marisnigri*, increasing concentrations of *M. acetivorans* with cocultures (SRB and hydrogenotrophic methanogen) resulted in an increase of methane production. This is most likely due to the fact that *M. acetivorans* could utilize the methanol layer of crude glycerin and the acetate by-product of SRB oxidation of glycerol. In the case of *D. vietnamensis* and *M. marisnigri*, a smaller concentration of *M. acetivorans* increased methane production, but higher concentrations had the reverse effect. However, when *D. vietnamensis* and *M. marisnigri* was grown on the supernatant of *M. acetivorans* the coculture grew faster and produced more methane than when grown under normal conditions (fresh media). The same was true for growth of *D. alcoholvorans 6133* and *M. marisnigri* on the supernatant. This suggests that the presence of *M. acetivorans* cells somehow affects the

coculturing between *D. vietnamensis* and *M. marisnigri*. Although, *D. alcoholvorans 6133* and *M. maripaludis* and *D. vietnamensis* and *M. maripaludis* grew fine and produced much more methane in the presence of *M. acetivorans*, neither culture grew very well on the *M. acetivorans* supernatant. Both cultures produced less methane and grew to a much lower optical density compared to growth under normal conditions (fresh media) on crude glycerin. Perhaps *M. acetivorans* produced a high concentration of toxins that inhibited the coculturing of the SRB with *M. maripaludis*. *M. acetivorans* could have also used up an important nutrient within the media that is essential for *M. maripaludis*. It appears that something is affecting the coculturing process and not necessarily the SRB or hydrogenotrophic methanogens since they have all shown to be capable of growing either in the presence or on the supernatant of *M. acetivorans*. The results suggested that the use of *M. acetivorans* for methanol and acetate utilization was feasible, but *M. maripaludis* and *M. marisnigri* responded differently.

Conclusions.

Experimentally, SRB and methanogen cocultures have displayed the capability of converting the waste product, crude glycerin, into a promising alternative energy. Future studies should involve gaining a deeper understanding of the complex nature of the microbial interactions between the SRB, hydrogenotrophic methanogens, and the acetoclastic methanogen, *M. acetivorans*. In order to overcome the slightly toxic nature of crude glycerin and to increase methane yield, a mixed consortium should be grown in a reactor as a biofilm and tested for increased tolerance and production.

Overall, *D. alcoholvorans 6133* and *M. maripaludis* produced more methane on crude glycerin than all other co/triculture combinations. *D. vietnamensis* and *M. maripaludis* and *D. alcoholvorans 6133* and *M. marisnigri* produced more methane than all other co/tricultures on fractionated glycerin. In general, methane production did not correlate with high growth rates. The coculture, *D. alcoholvorans 6133* and *M. marisnigri,* and triculture, *D. vietnamensis, M. maripaludis,* and *M. marisnigri,* grew the fastest of all co/tricultures on crude glycerin. *D. vietnamensis* and *M. maripaludis* grew the fastest on fractionated glycerin. The best crude glycerin conversion was seen when high concentrations of *M. acetivorans* were combined with: *D. vietnamensis* and *M. maripaludis*, *D. alcoholvorans 6133* and *M. marisnigri,* and *D. alcoholvorans 6133* and *M. maripaludis.* Approximately eight times more methane was produced when *M. acetivorans* was added to the cocultures, *D. vietnamensis* and *M. maripaludis* and *D. alcoholvorans 6133* and *M. marisnigri,* compared to growth without the acetoclastic methanogen.

Based upon both theoretical and observed yields, biological conversion of glycerin to methane is a feasible, safe, and a transformative mechanism to provide different uses for a biodiesel by-product that can improve overall economics and create an additional biofuel. A single approach will not satisfy all requirements when considering raw materials, region, or volatile energy and chemical markets; therefore, multiple strategies should be pursued to provide private and commercial entities options when selecting processing options in the biofuel industry. The described work has identified and performed initial characterization of microbial consortia that can tolerate

and convert glycerin to biogas without the need for glycerin purification. The ability to utilize glycerin as a feedstock for microbial conversions will circumvent industrial purification processes and will possibly alleviate price constraints for the biodiesel market.

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