AC 2011-228: DEMONSTRATING MICROBIAL GROWTH AND METABOLIC KINETICS WITH METHANOTROPHIC BACTERIA: A CLASSROOM LABORATORY EXPERIMENT

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Demonstrating Microbial Growth and Metabolic Kinetics with Methanotrophic Bacteria: A Classroom Laboratory Experiment

Abstract

With the field of biotechnology rapidly expanding, it is increasingly important to provide relevant, hands-on education to environmental engineering students in the area of microbial growth and metabolic kinetics. Students enrolled in introductory microbiology courses can have difficultly visualizing the physical growth of bacteria, the stoichiometric ratios of substrate consumption, and the results of metabolic processes that produce end products. Methanotrophic bacteria can be used as a model, allowing students in an experimental laboratory class to understand microbiological fundamentals. Methanotrophs have been well studied and many strains are available for purchase through American Type Culture Collection (ATCC) and other culture collection services. Methanotrophs are also ubiquitous in nature and can be isolated from many different environments, including soils and aqueous sediments. Additionally, methanotrophs possess a relatively simple and easily understandable metabolic pathway, consuming methane (electron donor) and oxygen (electron acceptor) as their primary substrates, and producing carbon dioxide and water as end products. The objective of this study was to develop a simple experiment that clearly shows students the concepts of microbial growth, and the stoichiometry associated with substrate consumption, using simple techniques with a readily available source of bacteria, i.e., methanotrophs. The laboratory study is conducted as follows: in sterilized serum bottles, methanotrophic bacteria in the exponential growth phase are inoculated in a Milli-Q water-based medium containing nitrate, other key nutrients (P, K, Na, Mg, Ca, etc.), and selected trace metals. The primary substrates, methane and oxygen, are then introduced and each bottle is placed on a shaker-table for incubation. Gas samples are periodically withdrawn from the headspace of each bottle and manually injected into a gas chromatograph to measure the concentrations of methane, oxygen, and carbon dioxide. This allows students to monitor the amount of substrates consumed and the amount of end product produced at different time points. Students can also track microbial growth by measuring the increase in turbidity of the medium via changes in optical density (OD). Results of the substrate consumption and carbon dioxide production are then plotted in a spreadsheet program (e.g., Excel). Typical results clearly depict the different rates of substrate consumption for oxygen and methane, and the rate of carbon dioxide production. This unique approach allows students to see firsthand the stoichiometry of the chemical reactions associated with the metabolism of metanotrophs. The results also clearly depict the microbial growth concepts of lag, exponential growth, and, if the experiment is continued long enough, microbial death. This experiment has been successfully conducted by both graduate and undergraduate students with several different genuses of methanotrophic bacteria including *Methylobacter, Methylonsinus,* and *Methylocystis,* indicating that any genus of the methanotroph family can be successfully grown under these

conditions. After using this approach, students will have a better understanding of microbial growth kinetics and the stoichiometry associated with the chemical reactions in the methanotrophic metabolic pathway, while gaining additional relevant lab experience.

Introduction

The field of Environmental Biotechnology utilizes microorganisms to protect and/or improve the quality of the environment. While people have understood the existence of bacteria for centuries, only recently have practices developed that employ bacteria to improve the environment. Some of these practices include preventing the discharge of pollutants, remediation of contaminated environments, and resource generation.[6] One bacterium that has been well studied in recent decades for its ability to degrade chlorinated solvents, such as trichloroethylene, dichloroehtylene, and vinyl chloride are methanotrophic bacteria. Methanotrophic bacteria are ubiquitous in nature and can be found in abundance at oxic-anoxic interfaces, where oxygen concentrations are low and methane concentrations are relatively high.[4,7] Examples of these locations include rice paddies, muddy soils, swamps, marshes, surface sediment in ponds and lakes, forest soil, and drainage water.[3,4] Methanotrophs are also critical to the natural environment because they regulate the biogeochemical methane cycle.[4]

Recently, methanotrophic bacteria have been the subject of study at Stanford University due to the ability of some strains to produce polyhydroxybutyrate, which is a polyester used by many bacteria as a carbon and energy source.[2] The following experiment was derived from a dynamic laboratory class associated with this work, and is useful for observing the physical growth of bacteria and associating that growth with the stoichiometric ratios of substrate consumption. Methanotrophic bacteria possess a simple and easily understandable metabolic pathway, consuming methane (electron donor) and oxygen (electron acceptor) as their primary substrates, and producing carbon dioxide and water as end products. The following methodology outlines a simple experiment that clearly shows students the concepts of, and the stoichiometry associated with, substrate consumption using simple techniques with a readily available source of bacteria.

Methodology

This laboratory study uses a batch study methodology. The following equipment is required:

- Gas Chromatograph (GC) capable of analyzing the following gases: O_2, CH_4, and CO_2. This study used a GOW-Mac GC (GOW-Mac Instrument Co., Bethlehem, PA) equipped with TCD detector with a CTR1 column (Alltech Associates Inc., Deerfield, IL) with helium carrier.
- Shaker table capable of continuously operating at 150 RPM.
- 158-ml serum bottles with rubber butyl stoppers and aluminum crimp caps
- Access to an autoclave
- Laboratory vacuum pump

- Needle and gas-tight syringe capable of withdrawing 0.5 mL of gas sample and manual injection into the GC
- 4 liters of prepared fluid medium for bacteria growth
- Source of methane gas and oxygen gas
- Gas standards for O_2, CH_4, and CO_2
- Lab notebook

Pre-Lab Preparation

Methanotrophic bacteria can be obtained from several sources including the American Type Culture Collection (ATCC), or other culture collection services. Methanotrophic bacteria can also be isolated from environmental conditions as described in Whittenbury *et al*, 1970, and Patt *et al*, 1974.[5,7] Methanotrophs should be appropriately stored prior to the experiment. Storage at 4^{o}C is appropriate if the experiment will be conducted within 72 hours. For longer durations, the bacteria should be stored at -20^{o}C.

The instructor, or teaching assistant, will prepare the fluid medium for bacterial growth. The medium will be prepared in a sterilized 2-liter glass container using Milli-Q water as the solvent. The following chemicals are added to the solvent: 0.8 mM $MgSO_4$, 10 mM $NaNO_3$, 0.14 mM $CaCl_2$, 1.2 mM $NaHCO_3$, 2.35 mM KH_2PO_4, 3.4 mM K_2HPO_4, 20.7 µM Na_2MoO_4, 10 µM FeEDTA, and 1 mL of a trace element solution [which contains, mg per liter: $FeSO_4 \cdot 7H_2O$, 500 mg; $ZnSO_4 \cdot H_2O$, 400 mg; $MnCl_2 \cdot 7H_2O$, 20 mg; $CoCl_2 \cdot 6H_2O$, 50 mg; $NiCl_2 \cdot 6H_2O$, 10 mg; H_3BO_3, 15 mg; EDTA, 250 mg]. Each 158-mL serum bottle used during the experiment will contain 50-mL of the fluid medium. Prior to inoculation of the medium, each serum bottle needs to be autoclaved to ensure sterility.

Thirty-six hours prior to the start of the laboratory, the bacteria needs to be re-grown to ensure the methanotrophs are in the exponential growth phase for the experiment. The bacteria should be re-grown in 158-ml serum bottles using 50-ml of the fluid medium to an $OD_{670} \geq 0.4$, as explained in Steps 1 and 2 of the *Lab Execution* section of this paper. Once an $OD_{670} \geq 0.4$ is reached, the bacteria are ready to be transferred to fresh 158-ml serum bottles for the laboratory execution.

Prior to the start of the laboratory itself, the instructor should break students into teams of two. Each team will need the following items: two 158-ml serum bottles, 100 mL of fluid medium, 2 rubber butyl stoppers, 2 aluminum crimp caps, one syringe and needle capable of withdrawing 0.5 mL of gas sample, and one lab notebook.

Student Training

Prior to lab execution, students need to receive a period of instruction on how to manually inject 0.5 mL of gas sample from the headspace of each 158-mL serum bottle into the GC. The execution will vary based on the type of GC used during the lab; however, the period of

instruction should include at least two practice iterations where students remove gas samples from the bottle's headspace and manually inject them into the GC. Failure to practice this step can lead to broken or bent needles, or, even worse, release of the gas headspace from the 158-ml serum bottle.

The instructor should demonstrate each step of the lab execution before the laboratory starts. Additionally, no student should operate the GC without prior instructor certification.

Lab Execution

Step 1 – Bottle Preparation: Each 158-ml serum bottle needs to be autoclaved prior to use to prevent contamination. Students will first add 50-mL of the pre-prepared fluid medium to the serum bottle. Once complete, the bottle will be capped with a butyl-rubber stopper and crimped with an aluminum crimp cap. Students will then remove the headspace gas in the 158-mL serum bottle and create a vacuum condition by connecting the headspace of the bottle to a vacuum pump. To ensure complete removal, the bottle's headspace needs to be under a vacuum for 1 minute. Upon completion of the vacuum, students will add 55 mL of methane gas and 55 mL of oxygen gas to the bottle's headspace with a plastic syringe. Each gas is taken from its respective gas cylinder to desired volume under standard atmospheric pressure. The addition of these quantities ensures that the bottle's headspace is approximately atmospheric pressure and that there is a 50/50 ratio of substrates available to the bacteria. Each student group should create two 158-mL serum bottles for testing. Creating two bottles will provide redundancy in the event that one of the bottles is compromised during the experiment.

Step 2 – Transfer of Methanotrophic Bacteria to 158-ml Serum Bottles: After the substrate gases are added, students will immediately inoculate the methanotrophs. The instructor should facilitate transfer from the 158-mL serum bottles of re-grown methanotrophs to the student's bottles to ensure there is no error during the transfer. 2.0 mL of culture (methanotrophs and fluid medium) should be transferred via a disposable 5.0 mL plastic syringe and 28G needle. Care should be taken not to release the headspace in the student's serum bottle (55 mL of each methane and oxygen) during the process. If the headspace is released, then the bottle should again be subject to vacuum and the gaseous substrates re-added.

The transfer of the methanotrophic bacteria marks the first time point in the experiment, and a 0.5 mL gas sample from the headspace will be immediately injected into the GC (procedure outlined in Step 3, *Sampling of the Gas Headspace*). Upon completion of the first injection, the bottle will be incubated on a shaker table operating at 150 RPM and at room temperature. Shaking the bacteria ensures that there is adequate mass transfer of the gas substrates to the fluid medium.

Note: if multiple students are conducting this experiment and there are a limited number of available GCs, then the instructor will have to establish a schedule to stagger group start times.

Step 3 – Sampling of the Gas Headspace

Once placed on the shaker table, the methanotrophic bacteria will immediately begin to consume the gaseous substrates, methane and oxygen. While the rate of substrate consumption depends on the species of methanotroph used, in general, the oxygen in the headspace will be depleted within 18 hours. Methanotrophs consume oxygen faster than methane as described by their metabolic stoichiometry, which is shown in the following reaction:

$$CH_4 + 2O_2 \rightarrow 2H_2O + CO_2$$

To allow for complete consumption of the methane in the gas headspace, additional oxygen (30 mL) will, therefore, need to be added at one of the later sampling points. At least 4 sampling points should be conducted during the 24 hour period to clearly depict the rates of gas consumption and production when graphed.

At each sampling point, the 158-mL serum bottles should be removed from the shaker table and a 0.5 mL gas sample from the headspace taken. Care should be taken to ensure that exactly 0.5 mL of gas sample is taken and injected immediately to the GC to prevent error. Once the gas sample is taken, the serum bottle should be immediately replaced on the shaker table. Gas standards for methane, oxygen, and carbon dioxide should be run at each sampling point to calibrate the data. If no gas standards are available, the laboratory can still be completed; however, the exact amount of gas substrate consumed can only be approximated. Note: only one student should inject samples from each 158-mL serum bottle and the gas standards at each sampling point. This will minimize human error due to differences in each student's injection technique.

Upon completion of the GC's run, students should record the time of sampling and the data reported by the GC for quantities of methane, oxygen, and carbon dioxide. It is likely that the GC will report data in terms of area counts or area under a curve. Students need to record the reported areas for each of the three gases from each 158-mL serum bottle, and the areas for the gas standards for comparison.

Step 4 – Graphing Substrate Consumption and Carbon Dioxide Production

The experiment will conclude when the methanotrophs in each 158-mL serum bottle consume all available methane. Students should use a spreadsheet program, such as Excel, to graph the data reported by the GC. First, the student must normalize the data by comparing it to known standards. Comparison to known standards will allow the student to determine the exact quantity of gases existing in the headspace at the time of sampling. Table 1 shows an example of how to tabulate data in Excel. The data in Table 1 corresponds to the graph in Figure 1.

Table 1: Data from a sampling iteration conducted with *Methylosinus trichosporium* OB3b, a Type II methanotroph.

Days	CO_2 Area Count	CO_2 (mL)	O_2 Area Count	O_2 (mL)	CH_4 Area Count	CH_4 (mL)	OD_{670}
0.00	5019.00	3.30	181052.00	50.53	145280.00	52.06	0.06
0.27	5321.00	3.44	151044.00	40.99	136032.00	48.28	0.09
0.45	13316.00	9.52	119625.00	31.50	107868.00	38.68	0.22
0.63	20943.00	15.35	51108.00	9.98	63044.00	22.80	0.35
0.78	22404.00	16.41	5253.00	0.00	36436.00	13.53	0.40
0.94	26322.00	19.95	128559.00	36.01	631.00	0.23	0.48

If no standards are available, the student can simply graph the data reported by the GC (usually in area counts) in Excel. While the exact quantity of gases will not be known using this method, the student can still clearly see substrate consumption and carbon dioxide production based on the changes in area of each gas.

Additional Tests: Optical Density, Total Suspended Solids, and Volatile Suspended Solids

Several additional tests can be conducted in conjunction with this laboratory study. This experiment measured the optical density (OD_{670}) at each sampling point using a Hach DR2800 Spectrometer. An 1.0 mL aqueous sample was withdrawn from the serum bottle using a disposable 5.0 mL syringe and 28G needle at each sampling point. The optical density was then graphed alongside substrate consumption to show the growth of the methanotrophic bacteria as the substrate was consumed. Biomass samples for VSS and TSS analysis can also be taken to measure the inoculation concentration prior to the laboratory. Procedures 209A, 209C, and 209D of *Standard Methods* should be used.[1] The biomass samples should be taken from the re-grown bacteria at the time of inoculation into the student's 158-mL serum bottles.

Results and Discussion

This procedure was successfully conducted by both graduate and undergraduate students as part of a dynamic laboratory study at Stanford University. Several different genuses of methanotrophic bacteria, including *Methylobacter, Methylonsinus,* and *Methylocystis,* were used during the experiment, indicating that any genus of the methanotroph family can be successfully grown under the outlined experimental conditions. Figures 1, 2, and 3 show graphs of the three species of methanotrophic bacteria used: *Methylobacter* (species unknown), *Methylonsinus trichosporium* OB3b, and *Methylocystis parvus* OBBP. As shown, each species of methanotroph completely consumed the oxygen within approximately 18 hours, and all of the methane within approximately 24 hours. At the point of complete methane consumption, between 13.6 and 20.5 mL of carbon dioxide, the end product, was produced. The graphs clearly depict the consumption of substrates and bacterial growth, allowing students to visualize the stoichiometry of the chemical reactions associated with the metabolism of methanotrophs. The results also

show the concepts of lag and exponential growth. Should the instructor choose to continue the experiment without adding additional methane and oxygen, the methanotrophs will show no metabolic activity and, within a matter of days, visibly decay.

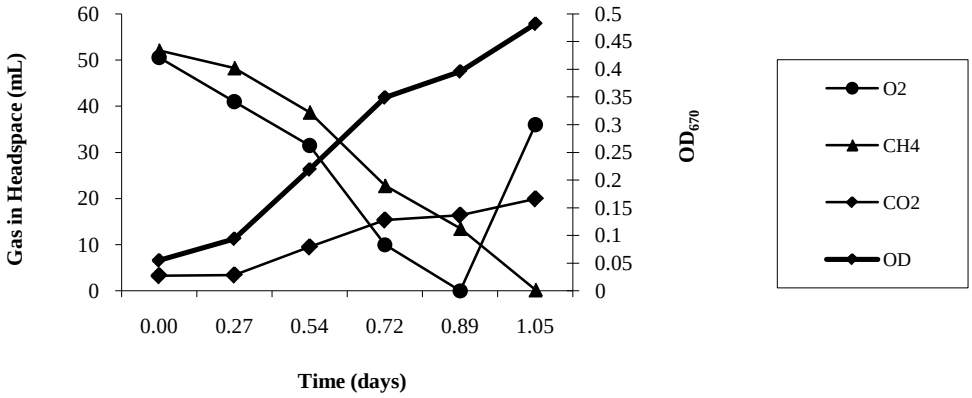

Figure 1: Substrate consumption, carbon dioxide production, and increase in optical density of *Methylonsinus trichosporium* OB3b over time. The spike in O_2 at 0.89 days indicates the addition of this substrate, which ensures that methane consumption will continue to completion.

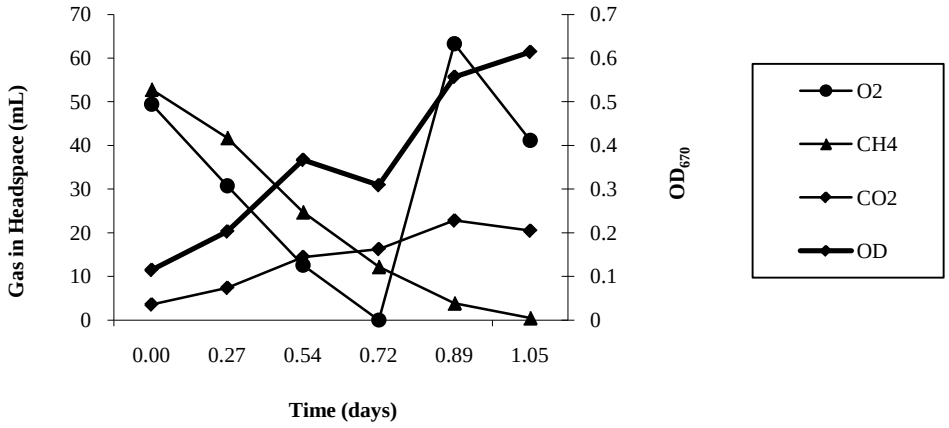

Figure 2: Substrate consumption, carbon dioxide production, and increase in optical density of *Methylocystis parvus* OBBP over time. The spike in O_2 at 0.72 days indicates the addition of this substrate, which ensures that methane consumption will continue to completion.

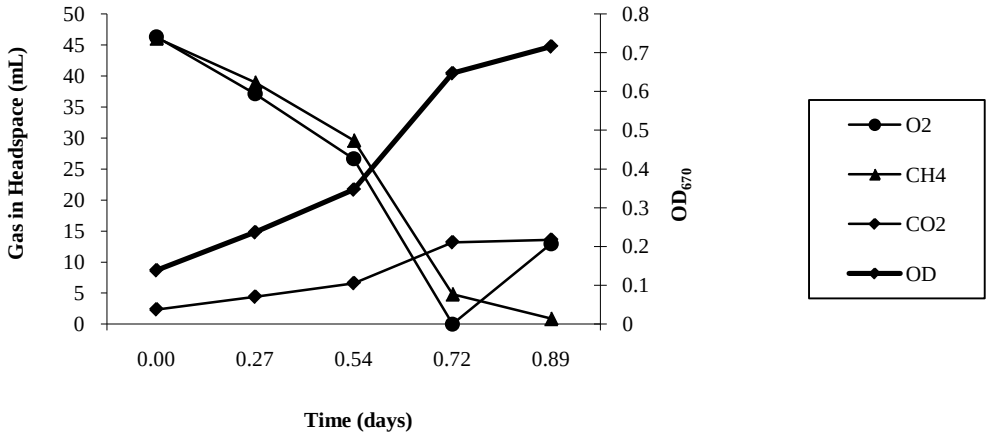

Figure 3: Substrate consumption, carbon dioxide production, and increase in optical density of *Methylobacter* (species unknown) over time. The spike in O_2 at 0.72 days indicates the addition of this substrate, which ensures that methane consumption will continue to completion.

The laboratory itself takes 2-3 days for the instructor to set-up, primarily to re-grow the bacteria, and approximately 24 hours for the students to execute. Therefore, the instructor will need to schedule the laboratory at a time when students are available to conduct sampling points approximately every 6 hours and can focus on proper execution of the lab. This laboratory is ideally suited for a class size of 12-15 students. However, the lab could be scaled up with an additional GC, more shaker table space, and the assistance of teaching assistants.

The laboratory class in which this experimental procedure was designed is a 3.0-credit, lab-based course that takes place at Stanford University once every two years and focuses on current topics in applied microbiology. Each class is unique; therefore no student assessment data is currently available. The laboratory will best support ABET Engineering Criteria Program Educational Outcome B, "an ability to design and conduct experiments, as well as to analyze and interpret data."

Student Evaluations

Upon completion of this laboratory, students should write their findings in a scientific paper format. One report should be generated per lab group (2 students). Students should create graphs similar to the ones presented in this paper, which will allow them to clearly see the metabolic activity and the metabolic kinetics of the methanotrophic bacteria.

Conclusions

Methanotrophic bacteria possess a simple and easily understandable metabolic pathway, consuming methane (electron donor) and oxygen (electron acceptor) as their primary substrates, and producing carbon dioxide and water as end products. The objective of this study was to develop a simple experiment that clearly shows students the concepts of microbial growth, and the stoichiometry associated with substrate consumption, using simple techniques with a readily available source of bacteria. Through this experiment, students will gain hands-on experience working with bacteria and seeing, first-hand, the stoichiometry associated with substrate consumption and end product production. These characteristics will likely make this lab more valuable than traditional bacterial growth labs, such as those involving *Escherichia coli*, to some educators.

References

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