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Development and comparisons of efficient gas-cultivation systems for anaerobic carbon monoxide-utilizing microorganisms

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Abstract

We describe a system for the cultivation of gaseous substrate utilizing microorganisms that overcomes some of the limitations of fixed volume culture vessels and the costs associated with sparging. Cali-5-Bond™ gas-sampling bag was used as the culture vessel. The bags contain approximately six times more mass of CO than the 40 mL vials at 1 atm of pressure and performed equally to the 40 mL vials in terms of their ability to maintain the composition of the gas over extended incubation times. Experiments using *Clostridium ljungdahlii* and CO as the sole carbon and energy source in both the gas sampling bag cultivation system and the traditional vial system demonstrated that this culture had a 15× increase in optical density in 24 h of incubation. The gas-sampling bags offer a viable alternative to gas sparging while overcoming the limitations of fixed volume culture vessels.

Keywords: Anaerobic microorganisms; Cultivation; CO; CO_2; H_2; Sparging; *C. lungdahlii*; Culture vessels

1. Introduction

The earth contains a wide variety of carbon reservoirs that can be harnessed to meet the societal power requirements in the form of gaseous, liquid, and solid fuels with liquid fuels being of most importance. A renewable source of fuels is required for meeting the future energy needs of the United States and the world.

A renewable source feedstock from which liquid fuels can be produced is biomass. Traditional biomass derived fuels such as ethanol utilize only a small percentage of available biomass because these processes are limited to the starches and sugar monomers contained within the fruit of the plant. Sugars contained in the rest of the biomass are in the polymerized form of cellulose, lignocellulose, and hemicellulose. These sugar polymers can be fermented only after an expensive depolymerization process such as acid hydrolysis or through enzymatic action with cellulases. An alternative to the depolymerization of the cellulose and hemicellulose is gasification of the biomass. Gasification of the biomass produces a synthesis gas (also known as syngas). Gasification is a thermal process that converts biomass, including lignocellulosic material, into synthesis gas (Maschio et al., 1994). Syngas is composed of varying amounts of CO, CO_2, H_2, CH_4, and trace amounts of S (Phillips et al., 1993).

Anaerobic microorganisms that utilize gaseous compounds such as CO, CO_2, and H_2 as their source of carbon and energy are known as acetogens and methanogens. The cultivation of these gaseous-utilizing-microorganisms is traditionally carried out by either displacing the headspace above or bubbling gases through the medium in a fixed

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volume culture vessel such as test tubes or flask and stoppered with a butyl-rubber stopper, as was the case with Ohwarki and Hungate (1977), Svetlitchnyi et al. (2001), and Sokolova et al. (2002). A similar technique to the one used here was described by Kafkewitz et al. (1973) for the cultivation of H_2-producing *Ruminococcus albus*. In their study, a chemostat was modified to include a football bladder for the collection of the H_2 produced. Cultivating microorganisms in this fashion requires a great deal of safety precautions especially when CO, a toxic substance, or H_2, a flammable substance, is the gas being sparged through the reactors. Henstra and Stams cultivated their CO-utilizing microorganisms, *Carboxydothermus hydrogenformans* and *Thermoterrabacterium ferrireducens*, using 120 mL bottles stoppered with butyl rubber and pressurized to ~1.7 atm with a mixture of CO_2 and either CO, N_2, or H_2 (Henstra and Stams, 2004).

Cultivation of anaerobic microorganisms requires specialized techniques from those used to culture aerobic microorganisms. Obviously, oxygen in the system must either be reduced to a level acceptable by target microorganisms or removed from the system. Doan et al. (1999) evaluated the Coy chamber, GasPak system, and Anaero-Pack system for maintaining anaerobic conditions. Results of their study did not show any appreciable difference in growth of the seven cultures tested. These types of systems work well for cultivation of anaerobic microorganisms that utilize carbon sources that are liquid or solid at room temperature and pressure. However, when a carbon source is in the gaseous form, cultivation of these microorganisms becomes more difficult.

Although these techniques have been successfully used to cultivate many types of microorganisms, there are limitations and concerns associated with these methods. Only a defined amount of substrate can be delivered at a time using fixed volume culture vessels with limited pressurizing capacities. Then, as the substrate is transferred into the liquid phase and consumed by microorganisms, its partial pressure decreases, and consequently, the mass transfer rate. Fast reactions could become mass transfer limited as pressure in the culture vessel decreases. Use of mass transfer limited data to calculate reaction kinetics (e.g., reaction order, rate constant, and activation energy) results in incorrect values for industrial scale-up of chemical and biological processes.

A new method of cultivation that operates at atmospheric pressure (safer to handle) and capable of minimizing or eliminating mass transfer limitation would add an important tool to the arsenal of techniques utilized by microbiologists with gas substrates. The system described below utilizes Cali-5-Bond™ gas-sampling bags to culture CO-, CO_2-, and H_2-utilizing microorganisms without substrate limitations and/or reduced substrate mass transfer rates experienced with fixed volume vessels. This system also decreases the high cost typically associated with vials sparged with the substrate.

2. Methods

2.1. Medium

All chemicals used in the medium were obtained in technical or laboratory grades from Fisher Scientific (Hampton, New Hampshire) or Sigma-Aldrich (St. Louis, Missouri).

The Acetate Production Medium (APM) utilized in this study was prepared as described in a patent by Gaddy and Clausen (1992). After autoclaving, the bottles were placed in an anaerobic glove bag (Coy Laboratory Products) where the medium cooled to room temperature and any dissolved oxygen was removed. Medium was dispensed into culture 40 mL Fisherbrand EPA vials (FisherScientific) using a Wheaton Adjustable Volume Self-Refilling Repetitive Syringe.

2.2. Culture vials

Fisherbrand 40 mL EPA vials were used with each of three types of caps: crimp, Si/PTFE, and Mininert®. Crimp tops (FisherScientific) included Teflon/Butyl stoppers. The Si/PTFE caps (FisherScientific) consisted of open-top polypropylene closures with 0.005'' PTFE/0.120'' silicone rubber septa with the PTFE side of the septum facing into the vial. The Mininert® valve caps from VICI Precision Sampling (Baton Rouge, Louisiana) included silicone needle septa with PTFE faced caps.

Tedlar HandyGrab™ gas-sampling bags were purchased from Zefon International, Inc. (Ocala, FL). The 0.6 L, 2 mil thick pillows (bags) featured one polypropylene fitting that combines valve and septum. Five mil thick, 0.5 L capacity teflon gas-sampling bags were purchased from Fisher Scientific. Each teflon bag had a septum fitting in a polypropylene housing. Cali-5-Bond™ gas-sampling bags were purchased from Calibrated Instruments, Inc. (Hawthorne, New York) and are shown in Fig. 1. The 5 mil thick, 0.5 L pillows were equipped with one replaceable septum holder and one twist on/off valve per bag. Si/PTFE septa were used in an all bags with the PTFE side facing the inside of the bag.

2.3. Headspace gas analysis

Headspace gases were analyzed using an Agilent 6890N Network Gas Chromatograph System with a Thermal Conductivity Detector and a manual injection port. The system used a column selection method fitted with two Supelco columns (Bellefonte, Pennsylvania): a 45/60 Molecular Sieve 5A (10 ft × 118 in Stainless Steel) and an 80/100 Porapak Q (6 ft × 1/8 in Stainless Steel). Calibration curves for N_2, O_2, and CO were prepared by analyzing known volumes of pure N_2, O_2, and CO samples purchased from Nexair in Columbus, MS using a Hamilton® 100 μL gas-tight sampling syringe.

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Fig. 1. Five mil thick, 0.5 L Cali-5-Bond™ pillows equipped with one replaceable septum holder and one twist on/off valve per bag.

2.4. Vial cap testing

This experiment was conducted to determine which of 3 septum types would perform best with respect to maintaining the headspace gas composition. A 1% resazurin in water solution was prepared for use as an oxygen indicator in this experiment. Resazurin solution reacts with oxygen, causing the solution color to change from colorless to pink when low levels of oxygen are present. Simultaneously boiling and sparging nitrogen into the solution prior to the experiment removed dissolved oxygen. Six vials were prepared using each of three types of vial cap: crimp tops, Si/PTFE screw caps, and Mininert® valve screw caps. Crimp tops with Teflon/butyl rubber stoppers were used with matching 20 mL vials, while the other caps were used with 40 mL vials. Vials were filled halfway with 1% resazurin solution (10 mL for crimp vials, 20 mL for others) in the anaerobic glove bag. All vials were capped with the appropriate tops and removed from the glove bag for gassing.

The vials with the septum materials to be evaluated were gassed with 100% CO using the technique described below. Gases were introduced into the vials using a sterile gassing syringe. The gassing syringe consisted of a pre-sterilized 10 mL glass syringe stuffed with glass wool with a rubber stopper on the plunger end. A glass tube inserted through a hole in the stopper connected the syringe to the tube leaving the gas mixing apparatus.

All gassing procedures were performed under a fume hood. Gas was allowed to flow into the sealed vial for 10–15 s to create a slightly positive pressure inside the vial and prevent oxygen from entering the headspace. At that time, the vial cap was loosened to purge the headspace of nitrogen. Gas flowed continuously into the vial for 2 min, and then the vial cap was tightened for the final 10–15 s to create a slight positive pressure within the vial headspace.

Three vials for each cap type were placed in the shaker incubator at 37 °C and shaken at 100 rpm. Three additional vials for each cap type were incubated statically at 37 °C incubator. Gas samples of all vials were taken every 24 h for 3 days with one final sampling after 7 days of incubation.

3. Results

3.1. Vial cap and culture vial evaluations

Determining the rate of gaseous substrate consumption by microorganisms on a microcosm scale, i.e. less than 100 mL, without the introduction of oxygen through a worn septum or substrate becoming rate limiting can be challenging. To this end a series of experiments was conducted to determine if septa enclosed 40-mL vials could over come these issues.

The first objective was to compare the ability of different septa to prevent loss of substrate and intrusion of oxygen from the atmosphere over time. In this experiment, three septa were evaluated. Teflon/Butyl septa (Fig. 2) performed well in the first 24 h of the experiment. It should be pointed out that error bars are magnified by the short *y*-axis (2%). The reason for the short *y*-axis was that using a full scale would not have allowed for viewing differences. However, subsequent headspace samplings at 48, 72 and 168 h post setup showed a steady increase in the oxygen concentration in the septa enclosed vials. Another septum evaluated was a Si/PTFE rubber septum. From the data,

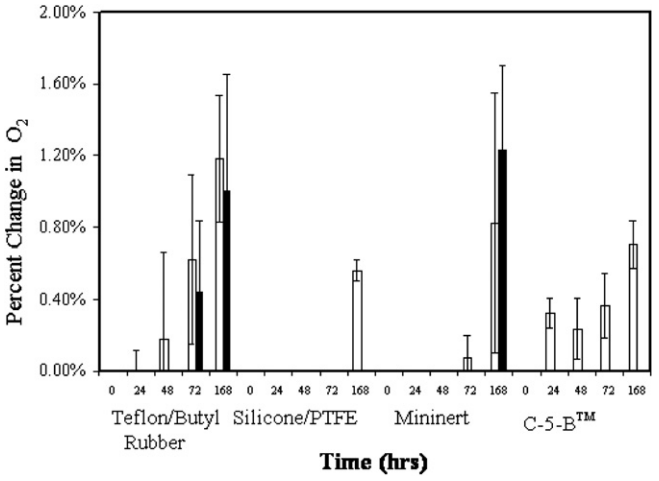

Fig. 2. Comparison of four capping materials used to prevent O_2 from entering the vessel over 168 h of incubation. Symbols: black bars, static incubation conditions; white bars, shaken incubation conditions.

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the ability of these septa to maintain anaerobic conditions over 72 h is clearly demonstrated for both incubation conditions. This type of septum was able to maintain strict anaerobic condition for 168 h when vials were allowed to remain static. Some oxygen (>1%) was present in samples taken after 7 days of incubation for those septa enclosed vials shaken at 150 rpm. Mininert® tops were the third type of closures evaluated. They outperformed the Teflon/Butyl rubber tops and performed similar to Si/PTFE rubber septum over a 3 day incubation period independent of the incubation conditions, i.e. shaken at 150 rpm or static. Headspace analysis on the vials closed with Mininert® tops after 168 h of incubation revealed oxygen concentrations in these culture vessels had only changed approximately 1% for those vessels incubated statically or shaken.

In addition to 40-mL vials and cap types, 2 mil and 5 mil gas-sampling bags manufactured from Teflon, and 0.5 L, 5 mil Cali-5-Bond gas-sampling bags were evaluated. Neither of the gas-sampling bags manufactured from Teflon were able to maintain the CO concentration for 24 h and were therefore removed from any further evaluations. The 5 mil gas-sampling bags outperformed all of the vials tested using static incubating conditions. Gas-sampling bags when shaken, demonstrated an increase in oxygen content in as little as 24 h. However, oxygen levels remained at the 24 h level for the next 48 h as shown in the 72 h analysis. The 168 h headspace analysis revealed a slight increase in oxygen but the overall change in oxygen in the headspace was less than 1%.

Effects of incubating conditions can be seen from the data presented in Fig. 2. The 40 mL vials sealed with the Si/PTFE rubber septum or Mininert® tops and the gas-sampling bags statically incubated showed no detectable oxygen after 48 h of incubation and only a trace amount after 72 h of incubation for those vessels sealed with Mininert® tops. Incubation conditions appear to have little impact on vessels sealed with Teflon/Butyl rubber stoppers after 72 h of incubation. Although oxygen entered the gas-sampling bags that were shaken, these values remained constant after 72 h of incubation.

Changes in N_2 and CO over time for these culture vessels were also measured and are presented in Figs. 3 and 4. The differences in performance of the septa evaluated become apparent. As before in Fig. 2, short *y*-axis were used to demonstrate differences but give the appearance of large errors. The vials sealed with the Teflon/Butyl rubber and Si/PTFE rubber demonstrated changes in the concentration of N_2 and CO within 48 h of incubation for both the vials shaken and incubated static. Change in N_2 and CO for 40 mL vials sealed with Mininert® tops was less than 1% through 72 h of incubation. However, headspace analysis after 168 h of incubation showed that average changes in N_2 and CO ranged between 4–6% from 95% for N_2 and 5–7% from 100% for CO. Variations within these sample sets also increases sharply. Gas-sampling bags on the other hand showed less than a 1% change in N_2 for all sampling times and incubation conditions. Change in CO was approximately a 1% change for all sampling times. Those gas-sampling bags that were shaken during incubation had the greatest change in CO after 24 h of incubation but maintained the CO for the duration of the experiment.

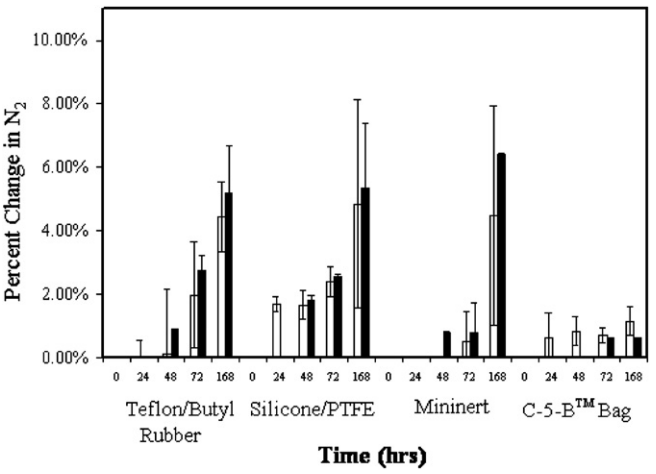

Fig. 3. Comparison of four capping materials used to maintain an atmosphere of N_2 over 168 h of incubation. Symbols: black bars, static incubation conditions; white bars, shaken incubation conditions.

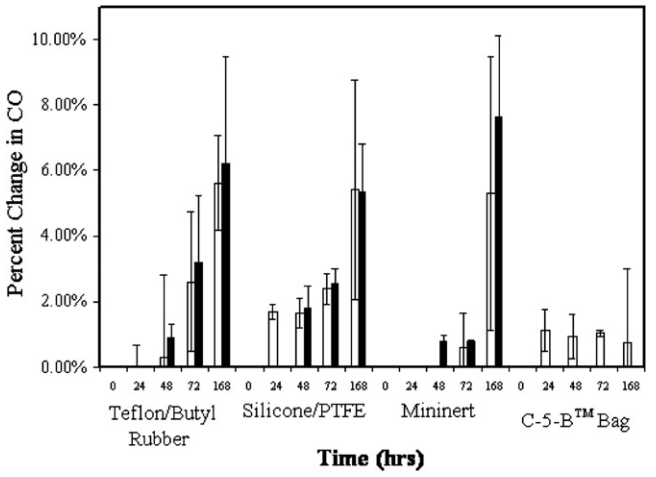

Fig. 4. Comparison of four capping materials used to maintain an atmosphere of CO over 168 h of incubation. Symbols: black bars, static incubation conditions; white bars, shaken incubation conditions.

3.2. Mass determination

Unlike highly water-soluble substrates like sugars and peptides, CO is not very water-soluble and the mass that can be applied is limited by the headspace of the culture vessel. For example, a 40 mL vial with 20 mL of cell suspension is limited to 20 mL of CO at 1 atm of pressure. Obviously, pressurizing the 40 mL vials can increase the mass of CO and its water solubility in these fixed volume vials. Unfortunately, there is little information available as to the amount of pressure that can be safely applied to these types of vials.

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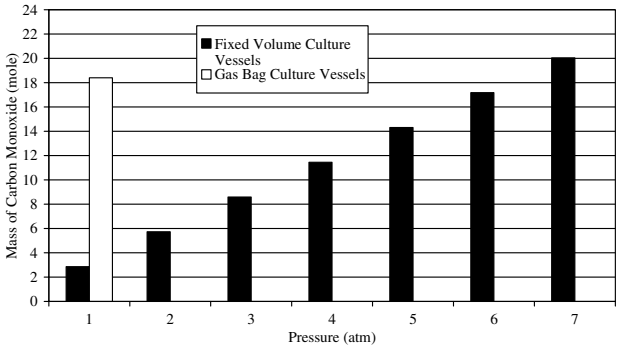

Fig. 5. The effects of pressure on the mass of CO in fixed volume vessels compared to the mass of CO in a gas-sampling bag. Symbols: white bar, Gas Bag Culture Vessel; black bars, Fixed Volume Culture Vessels.

Calculations were performed to determine what pressure of CO would be required in a 40 mL vial to equal the mass of CO in a 500 mL Cali-5-Bond™ gas-sampling bags assuming each had 10 mL of medium. Results of these calculations are given in Fig. 5. Data show that gas-sampling bags contain approximately 6 times more mass of CO than 40 mL vials at 1 atm of pressure. To achieve the same mass of CO in a vial that was in a gas-sampling bag, the vial would have to be pressurized to 6.3 atm with CO. Given that no information is available as to whether or not the vials could safely maintain these conditions, it would not be advisable to pressurize these 40 mL vials significantly above 1 atm. Pressurizing these vials could increase the loss of substrate and reduce the safety of the experiment.

3.3. Cultivation of CO-utilizing microorganism

The ability of a gas-sampling bag cultivation system to cultivate CO-utilizing microorganisms was evaluated using *Clostridium ljungdahlii* (ATCC # 55383) as the test organism and compared to a traditional vial system using the same microorganism with CO as the sole carbon and energy source. Results presented in Fig. 6 shows that those cultures in the gas-sampling bag cultivation system responded much quicker than those in the vials in less than 24 h as evidenced by the increase in absorbance. No difference was found for cultivation of *C. ljungdahlii* in the gas sampling bag cultivation system using shaken or static conditions. Cultures in the vials showed a slight increase in optical density readings. Absorbance of light at 600 nm wavelengths was measured for each sample via Spectronic * Genesys * 20 Spectrophotometer (Thermo * Electron) after 24 h of incubation. As with the gas sampling bag cultivation system, the vial data showed no effect of shaking the culture during incubation versus leaving it static in terms of increased optical density readings. The optical density readings for the gas sampling bag cultivation system were 15× greater than those for the vials after 24 h of incubation.

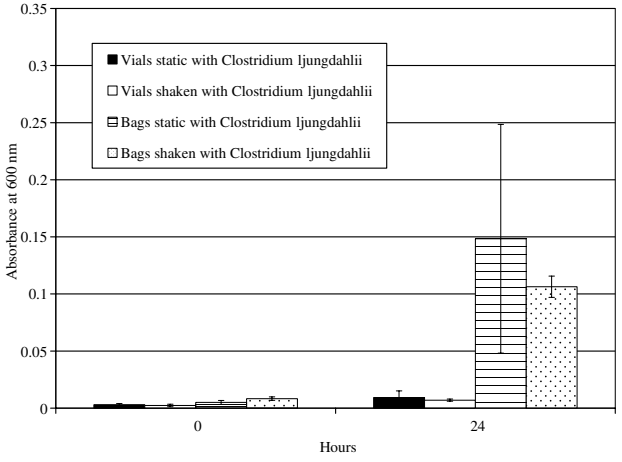

Fig. 6. A comparison of vials and gas-sampling bags for growing *C. ljungdahlii* on CO as the sole carbon and energy source while incubated statically and shaken.

4. Discussion

Cultivation of microorganisms that utilize gaseous carbon compounds as carbon and/or energy requires different techniques from those used to cultivate microorganisms metabolizing sugars, amino acids, or lipids. The difficulty of these techniques can be even more challenging when the culture requires an anaerobic environment. Often the microorganism's ability to metabolize these gaseous substrates is limited by an inadequate supply of substrate or mass transfer rate of substrate into the aqueous phase. This is due in large part to the types of culture vessels that are traditionally used (i.e. bottles, vials, and flasks). The use of gas-sampling bags is a novel method for cultivating microorganisms that utilize gaseous substrates. This method overcomes limited supply of substrate inherent with fixed volume bottles, vials, and flasks and performs as well as other more established techniques maintaining anaerobic conditions. Gas-sampling bags can be purchased in sizes ranging from 0.25 to 1.00 L, which gives flexibility in designing experiments. Depending on the gas-sampling bag design, these systems can be recharged with a gaseous substrate with little effort.

An additional benefit of gas-sampling bags is a larger driving force for substrate transfer from the gas to the liquid phase over the duration of the experiment. In fixed volume vessels, substrate rate of disappearance from the gas phase is given by:

$$\frac{dC_A}{dt} = r_A, \tag{1}$$

where C_A is the substrate concentration, $\frac{mol}{L}$, r_A is the rate of disappearance of substrate A, $\frac{mol}{L \cdot s}$.

In contrast, in the sampling bags the substrate rate of disappearance is given by:

$$r_A = \frac{dC_A}{dt} + \frac{C_A}{V}\frac{dV}{dt}, \tag{2}$$

where V is the head space volume, L.

As can be observed in Eqs. (1) and (2), the reduction in volume of the gas-sampling bags causes a faster substrate rate of disappearance. Gas-sampling bag systems favor kinetically limited condition compared to fixed volume vessels. This condition is critical to measure reliable kinetic parameters. In other words, as microorganisms consume substrate from the gas-sampling bags the volume reduction enhances substrate transfer into solution compared to fixed volume systems.

Sterilization and reuse of gas-sampling bags is an issue that could not be addressed by autoclaving in this study. It was observed that after autoclaving the 5 mil gas-sampling bags they were not capable of maintaining a headspace of CO due to micro-tears in the bags or damages to the seams. Bags could be washed to remove residues from a previous experiment but would need to be sterilized by means other than autoclaving such as ethylene oxide or γ radiation.

It should be pointed out that gas-sampling bags manufactured from Teflon used in this study failed to maintain CO in the bag for 24 h. The other systems evaluated were the 2 mil Tedlar gas-sampling bags and their performance was determined to be unacceptable. Based on the findings of this study it is suggested that gas-sampling bags manufactured with a thickness of 5 mil or greater be utilized to culture microorganisms on this type of gaseous substrate.

Cultivation experiments using *C. ljungdahlii* demonstrated the effectiveness of the gas sampling bag cultivation system to cultivate CO-utilizing microorganisms. This system produces a greater concentration of cells in 24 h of incubation than traditional fixed volume system produced. Wide deviation with the gas sampling bag cultivation system was from one sample at the 24 h readings with an optical density reading of 0.26 while the other two samples had optical density measurements of 0.104 and 0.078. A possible explanation to this observation is that a clump of cells was retrieved from the gas sampling bag cultivation system and summarily dispersed prior to taking the optical density reading. In all, the optical density readings were 15× greater than those observed for the *C. ljungdahlii* cultivated in the vials. This strongly suggests that the gas-sampling bag cultivation system is superior to traditional vial cultivation system for *C. ljungdahlii* using CO as the sole carbon and energy source.

This work has demonstrated the feasibility of using gas-sampling bags as vessels to cultivate gaseous substrate-utilizing-microorganisms. This system can overcome the supply limitations that are inherent with fixed volume vessels. Operation at atmospheric pressure and the capability of utilizing larger amounts of gas at the initiation of an experiment increases safety, minimizes handling to toxic gases, and enhances the reliability of the measured kinetic parameters.

Acknowledgements

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