Proposal Title: Interaction and Synergism of Microbial Fuel Cell Bacteria within
Methanogenesis

NASA GSRP Summary of Research (NAG 9-1555)

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In support of Master of Science Thesis in the Aerospace Engineering Sciences
Department at the University of Colorado:
"Characterization of the Effect of Butyrate on Hydrogen Production in
Biophotolysis for use in Martian Resource Recovery"

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# 1 Synopsis

NASA GSRP (NAG 9-1555) was used to support student Jackson Lee as part of a Master of Science Thesis in the Aerospace Engineering Sciences Department at the University of Colorado, titled: "Characterization of the Effect of Butyrate on Hydrogen Production in Biophotolysis for use in Martian Resource Recovery" which took place 9/2002-9/2004. The award allowed the student to focus solely on independent research in the area of biologically produced hydrogen and allowed unique collaboration within CU and with the Department of Energy's National Renewable Energy Laboratory.

Biological hydrogen production from waste biomass has both terrestrial and Martian advanced life support applications. On earth, biological hydrogen production is being explored as a greenhouse neutral form of clean and efficient energy. In a permanently enclosed space habitat, carbon loop closure is required to reduce mission costs. Plants are grown to revitalize oxygen supply and are consumed by habitat inhabitants. Unharvested portions must then be recycled for reuse in the habitat.

Several biological degradation techniques exist, but one process, biophotolysis, can be used to produce hydrogen from inedible plant biomass. This process is two-stage, with one stage using dark fermentation to convert plant wastes into organic acids. The second stage, photofermentation, uses photoheterotrophic purple non-sulfur bacteria with the addition of light to turn the organic acids into hydrogen and carbon dioxide. Such a system can prove useful as a co-generation scheme, providing some of the energy needed to power a larger primary carbon recovery system, such as composting.

Since butyrate is expected as one of the major inputs into photofermentation, a characterization study was conducted with the bacterium *Rhodobacter sphaeroides* SCJ, a novel photoheterotrophic non-sulfur purple bacteria, to examine hydrogen production performance at 10 mM-100 mM butyrate concentrations. As butyrate levels increased, hydrogen production increased up to 25 mM, and then decreased and ceased by 100 mM. Additionally, lag phase increased with butyrate concentration, possibly indicating some product inhibition. Maximal substrate conversion efficiency was 8.0%; maximal light efficiency was 0.89%; and maximal hydrogen production rate was 7.7 umol/mg cdw/hr (173 ul/mg cdw/hr). These values were either consisitent or lower than expected from literature.

This NASA GSRP will not be renewed for the following years. The author plans to join the peace corps in the Philippines for two years before resuming doctoral work. It is hoped in the future that he can work on advanced life support applications relating to environmental biotechnology.

## 2 Acknowledgements

## 3 Introduction to Carbon and Hydrogen Cycling

The main purpose of using a CELSS is to close the carbon cycle by growing edible crops from $CO_2$ and recycling a portion of the carbon residue into new edible crops. This will reduce the overall supply mass of the system. Essentially, the carbon processing system has two components, a plant growth and preparation component and a waste processing component. Carbon wastes typically consist of waste inedible biomass, exhaled $CO_2$ and $CO_2$ reserves, human wastes, and uneaten food particles and food preparation wastes. When studying mass flows in a CELSS, a large fraction of wastes predicted in models tend to be inedible waste biomass from plants for all mission scenarios involving plant growth, thus the current emphasis on biomass degradation and recovery of useful materials (Verostko, Joshi et al. 2002).
But the carbon cycle is not the only cycle that is required on a long term Martian base. Water is cycled conserve water consumables, but plants take in water and turn it into carbohydrates using the photosynthesis equation (Madigan, Martinko et al. 2003):

$$CO_2 + H_2O \xrightarrow{h\nu} (CH_2O) + O_2$$
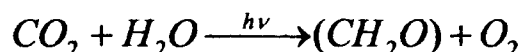
Waste carbon material therefore contains a significant fraction of hydrogen derived from spacecraft water reserves. Over time, inedible biomass can also become a drain on hydrogen (and therefore water) resources as well.

For example, in a wheat growth model from the ALS Baseline Values and Assumptions Document (Hanford, Ewert et al. 2002), for the extended mission case where all food crops are grown, the predicted total inedible biomass per crewmember-day is 1.271 kg/CM-d. Table 1.1 shows the ash analysis of the inedible portion of various crops grown at Johnson Space Center (JSC). Hydrogen accounts for about 5% of the total weight. Therefore, roughly 0.0633 kg/CM-

d of hydrogen or the equivalent of 0.572 kg/CM-d worth of water being lost in dry inedible wheat biomass

**Table 3.1 Ash Analysis of Various Crops grown hydroponically at JSC (Alazraki 2000)**

|  | Sample Type | | | |
|---|---|---|---|---|
|  | Potato Tops | Tomato Tops | Soy Tops | Wheat Tops |
| Wt % |  |  |  |  |
| Carbon | 30.81 | 30.54 | 38.99 | 36.32 |
| Hydrogen | 4.56 | 4.54 | 5.88 | 5.19 |
| Oxygen (by Diff) | 37.52 | 38.9 | 41.41 | 40.09 |
| Nitrogen | 3.91 | 4.19 | 3.12 | 3.04 |
| Sulfur | 0.61 | 0.77 | 0.36 | 0.67 |
| Ash | 22.59 | 21.06 | 10.24 | 14.69 |

For the Mars DRM (Hoffman and Kaplan 1997), the expected surface stay for 6 crewmembers is 619 days. Taking this as a baseline for an extended surface stay mission this comes to a loss of about 236 kg of hydrogen being sequestered in unprocessed inedible biomass if continuous wheat growth is conducted. This is the equivalent loss of 2124 kg of water from the water system just for wheat growth.

While the biological digestion processes such as SEBAC, anaerobic digestion, etc. outlined in the Waste Workshop of 2000 (Verostko, Joshi et al. 2002) can solve this problem by returning carbon material back to water and carbon dioxide, they involve the use of methane combustion, which in turn, requires a natural gas-powered generator system to be brought aboard. Such equipment have several disadvantages over fuel cell stacks. They utilize internal combustion to power an electric motor, and thus have issues with moving parts, lower efficiencies, noise, and flue gases when compared to fuel cells (ECER 2004). If gaseous hydrogen could be recovered directly, bypassing methane formation, this effluent can be sent to a hydrogen fuel cell to provide a form of energy cogeneration to power the machinery intensive (especially with composting) degradation process. Such a hydrogen recovery system can be a piggyback on the SEBAC or a composting system described in the Waste Workshop (Verostko, Joshi et al. 2002), or serve as a standalone technology coupled to a large mechanical composting device.


# 4 Literature Review of Biophotolysis

## 4.1 Overview

An emerging method of biological hydrogen production, called biophotolysis, or photofermentation, involves using certain bacteria under anaerobic conditions to produce hydrogen from decaying agricultural or industrial organic wastes (Sasikala, Ramana et al. 1990). In direct biophotolysis, photoautotrophic sulfur consuming eubacteria (algae), when deprived of sulfur in an anaerobic environment can produce molecular hydrogen for a limited time by splitting water directly. However, the process requires a rest phase, and also produces molecular oxygen, while being strictly anaerobic (Melis and Happe 2001).

In indirect biophotolysis, another kind of bacteria uses light (as an energy source) and waste carbon, typically in the form of organic acids, to produce hydrogen (Hallenbeck and

Benemann 2002). Indirect biophotolysis is a two-stage process very similar to the two-stage methanogenesis process (See Fig 2.1). As in methanogenesis, bacteria and fungi naturally present in the environment derive energy and produce some hydrogen by degrading dead waste organic matter into simple organic acids and alcohols. Such a situation takes place naturally in a waste treatment pond or compost heap and has been heavily studied (Bitton 1999). In the second stage, these organic acids are harvested and fed as a substrate to photoheterotrophic bacteria and more hydrogen is produced. Similarly, R. sphaeroides itself can be grown up and used as the organic matter in two-stage indirect biophotolysis (See. Fig. 2.8) (Hallenbeck and Benemann 2002).
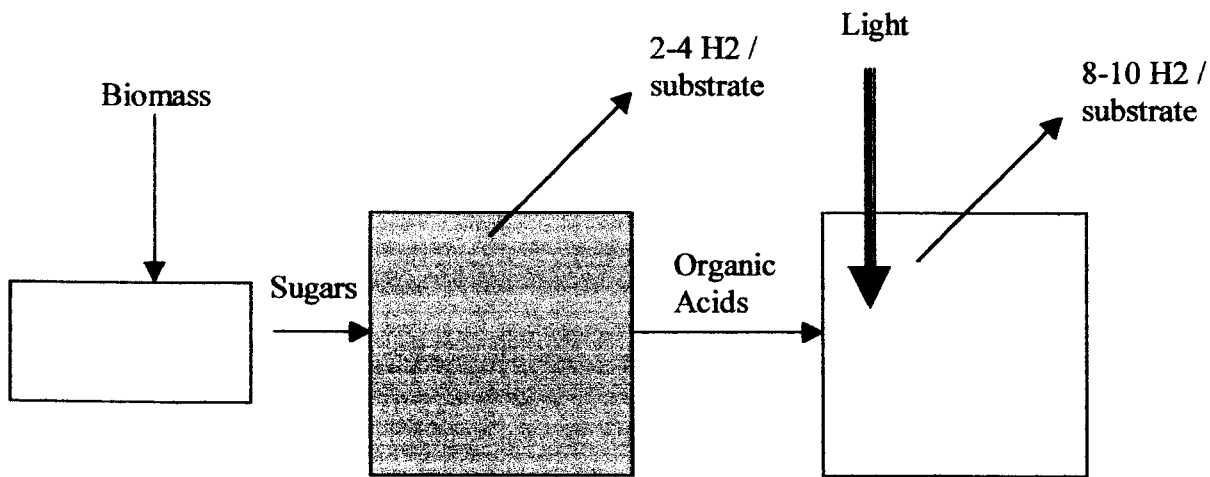


**Figure 4.1 Schematic of the Two-Stage Indirect Biophotolysis Process where waste biomass fermentation is used (Maness 2004b)**
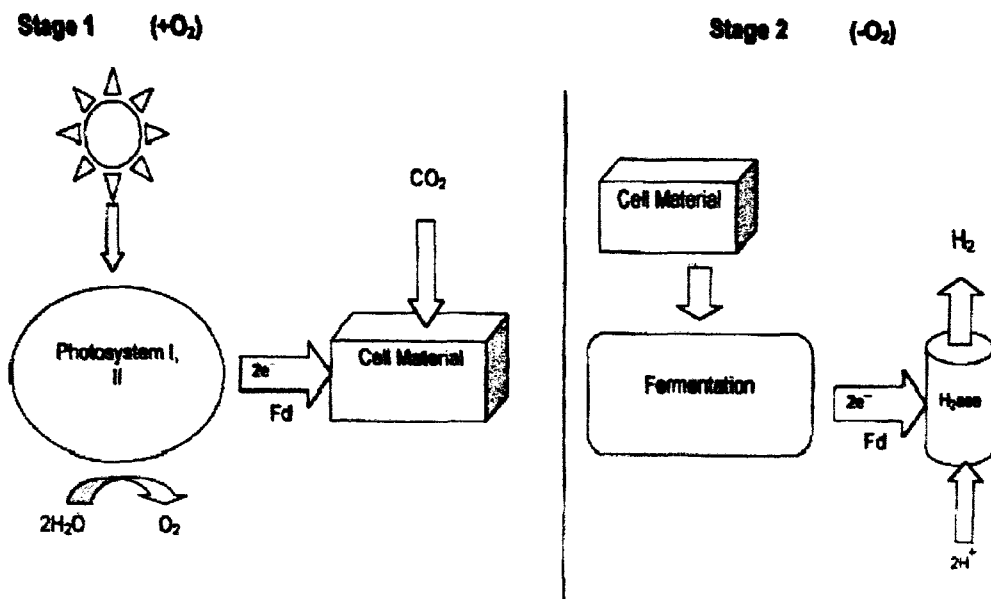
**Figure 4.2 Indirect Biophotolysis where cell mass is used (Hallenbeck and Benemann 2002)**

## 4.2 Microbiology, Energetics, and Biochemistry

A photoheterotrophic organism is one that typically takes energy in as light, but takes carbon in as organic substrates, rather than CO2 as in higher plants (Akkerman 2002). Photoheterotrophic organisms like *Rhodobacter sphaeroides* and other "non-sulfur purple bacteria" can take in organic acids and degrade them to CO2 plus H2. Theoretically 4-10 mol H2 per mol substrate can be produced, depending on the substrate (See Table 2.1 below) (Sasikala, Ramana et al. 1990). The redox schematic is shown below (Fig. 2.3). Organic acids lose electrons, which receive an energy boost from light. The electrons join with hydrogen ions from water to form hydrogen gas. Oxygen from water is used to oxidize carbon to carbon dioxide.



**Figure 4.3 Redox schematic of purple "non-sulfur" bacteria**

**Table 4.1 Theoretical Conversion of Organic Substrates to H2 (Sasikala, Ramana et al. 1990)**

Acetate: $C_2H_4O_2 + 2\ H_2O = 2\ CO_2 + 4\ H_2$

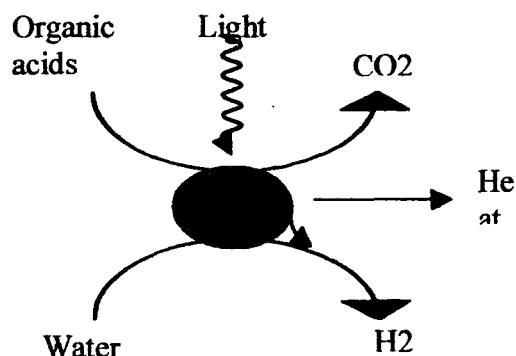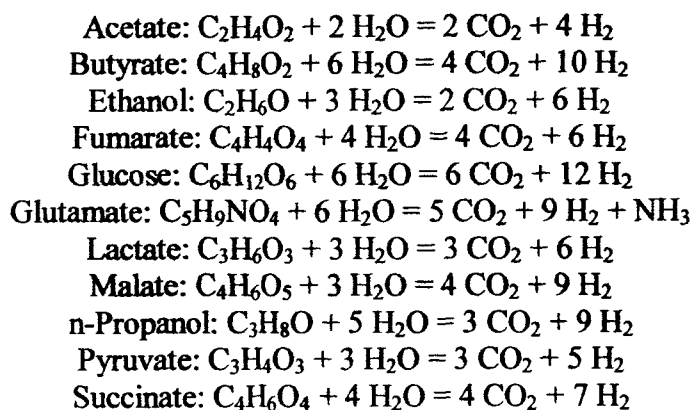Butyrate: $C_4H_8O_2 + 6\ H_2O = 4\ CO_2 + 10\ H_2$

Ethanol: $C_2H_6O + 3\ H_2O = 2\ CO_2 + 6\ H_2$

Fumarate: $C_4H_4O_4 + 4\ H_2O = 4\ CO_2 + 6\ H_2$

Glucose: $C_6H_{12}O_6 + 6\ H_2O = 6\ CO_2 + 12\ H_2$

Glutamate: $C_5H_9NO_4 + 6\ H_2O = 5\ CO_2 + 9\ H_2 + NH_3$

Lactate: $C_3H_6O_3 + 3\ H_2O = 3\ CO_2 + 6\ H_2$

Malate: $C_4H_6O_5 + 3\ H_2O = 4\ CO_2 + 9\ H_2$

n-Propanol: $C_3H_8O + 5\ H_2O = 3\ CO_2 + 9\ H_2$

Pyruvate: $C_3H_4O_3 + 3\ H_2O = 3\ CO_2 + 5\ H_2$

Succinate: $C_4H_6O_4 + 4\ H_2O = 4\ CO_2 + 7\ H_2$

## 4.3 Nitrogenase and the Photosystem

Purple non-sulfur bacteria are able to fix gaseous nitrogen using the nitrogenase enzyme, but this enzyme is also directly responsible for hydrogen evolution in the absence of nitrogen. During nitrogen assimilation, the overall energy consuming reaction is:

$$N_2 + 8e^- + 16ATP \xrightarrow{\ Nase\ } 2NH_3 + H_2 + 16ADP + 16P_i \quad \text{(Akkerman 2002)}$$
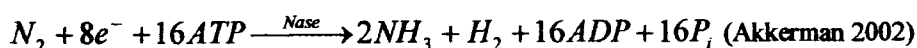
This ammonia is in turn assimilated into new proteins, including more nitrogenase (Sasikala, Ramana et al. 1990).



**Figure 4.4 The Nitrogenase Electron Transport Pathway (Akkerman 2002)**

When nitrogen no longer remains, nitrogenase can also consume ATP and NADH to form hydrogen.

$$NADH + H^+ + 5ATP \xrightarrow{Nase} 5ADP + H_2 + NAD + 5P_i$$
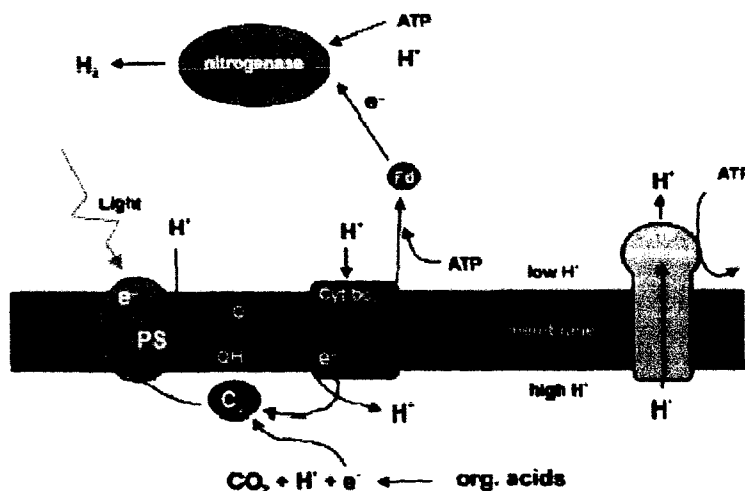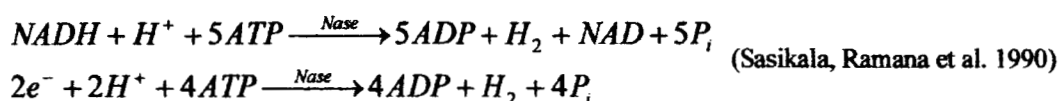$$2e^- + 2H^+ + 4ATP \xrightarrow{Nase} 4ADP + H_2 + 4P_i$$

(Sasikala, Ramana et al. 1990)

Nitrogenase functions as the end receptor (through ferrodoxin (Fd)) of the photosystem from light absorbing chlorophyll (See Fig 2.4). Light is absorbed and transfers its energy to electrons stripped from organic substrates. These electrons are used to pump protons, which in turn provide a gradient for ATP synthase. This photosystem is more primitive than photosystems typically found in higher organisms and has an absorbance range between 800-900 nm, as well as a few absorbance peaks in the 600 nm range (See Fig. 2.5 below) (Sasikala, Ramana et al. 1990). Nitrogenase is feedback inhibited by ammonium and oxygen so therefore hydrogen production is often run under inert gases such as argon and helium. Because of this feedback, nitrogen or nitrogenated substrates are often used as the limiting nutrient. At the same time, uptake hydrogenase can also harvest hydrogen under basic conditions so that no net hydrogen production is observed. Lastly, the specific carbon/nitrogen ratio also influences nitrogenase repression, and therefore the level of hydrogen production (Sasikala, Ramana et al. 1990).
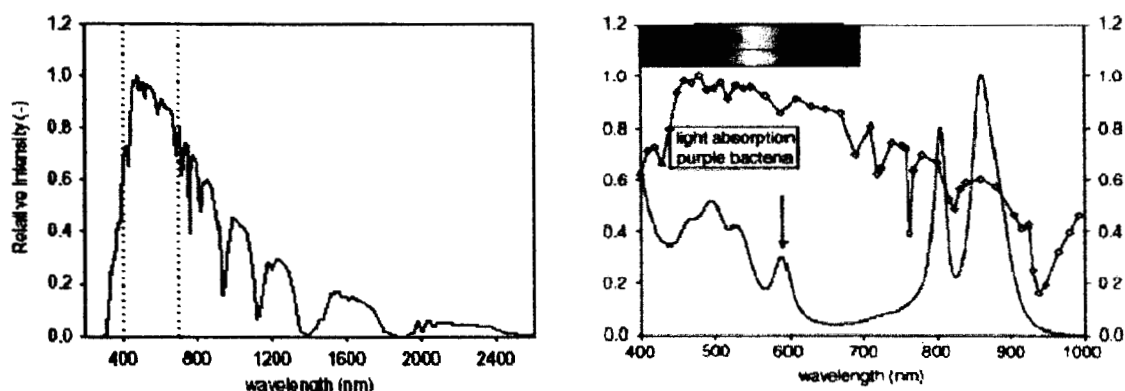


**Figure 4.5 Light spectrum of earth (left) and the absorbance peaks of purple non-sulfur bacteria (right) (Akkerman 2002)**

While much of the carbon used by photoheterotrophic bacteria is utilized in this manner, some of it is also redirected for carbon assimilation. Generally, this carbon is polymerized to poly-3-hydroxybutanoates and lost for hydrogen production. For highly reduced substrates (such as butyrate), CO2 is used as the redox electron *acceptor* and is expressed by:

$$2nCH_3CH_2CH_2COOH + nCO_2 \rightarrow 2(CH_3CH_2CHCOO^-)n + (CH_2O)_n + nH_2O$$ (Tabita 1995).
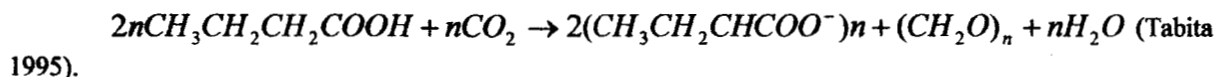
This strange behavior is an adaptation to the lack of a suitable anaerobic redox acceptor for metabolism. Photoassimilation products are therefore closely coupled to CO2 fixation in the Calvin-Benson cycle. Note, this indicates for many substrates, carbon dioxide or bicarbonate is necessary for *growth* (Tabita 1995) but not necessarily hydrogen evolution.

The substrate conversion efficiency can thus be expressed as a percentage of the stoichiometric maximum hydrogen production from Table 3.1 versus the observed hydrogen evolution and is stated as (Sasikala, Ramana et al. 1990):

% Substrate conversion efficiency = 100 x Observed H2 / Theoretical H2

Similarly, the light efficiency is typically expressed as the enthalpy of the hydrogen produced to the total energy of the light input into the reactor.

% light conversion efficiency = 100 x $H2_{enth}$ / $E_{light}$

## 4.4 Growth Conditions and Suitability for Mars

In the literature, various growth conditions have been tested. As stated previously, a carbon dioxide/ inert gas environment low in oxygen best suits these bacteria. Over the years, it has been shown that the best conversion efficiencies exist in low light (6.5-20 klux)(Sasikala, Ramana et al. 1990) low nitrogen (2-10 mM)(Maness 2004) environments with a pH of 6.7-7.5 (Sasikala, Ramana et al. 1990). Ammonium and glutamate were found to be the best nitrogen donors, and acetate was shown to have the highest substrate conversion efficiency (Sasikala, Ramana et al. 1990). The low light requirement stems from the self-shading effect found in photosynthetic bacteria. Photoheterotrophic bacteria are waterborne (brackish and fresh) organisms that have adapted to convert light best at low intensities. Above the light saturation point, excess photons are lost from the photosystem as waste heat (photocenter excitation and vibration) (Sasikala, Ramana et al. 1990). Much research in genetics has been done to explore improving this situation but no significant progress has occurred yet (Sasikala, Ramana et al. 1990). To counter this inability to work well in full sunlight, various reactor configurations have been tried, ranging from immersed light reactors, to flat panel reactors, to active mixing, with varied results and increased costs (Sasikala, Ramana et al. 1990).

While these faults exist terrestrially, they are actually somewhat of an advantage (or not applicable) for Mars based applications. Carbon dioxide or argon, principle chemicals of the Martian atmosphere, can be compressed for plant growth chamber air or redirected from any ISRU scheme. On earth, careful measures must be taken to ensure that no oxygen enters the system. Such a problem does not exist on Mars and greatly reduces the complexity of the system. Also, the ambient insolation on Mars is often less than a half to a tenth of that on earth, especially during a dust storm (See Fig. 2.6 below). As it so happens, this is near the intensities reported in literature for maximal light conversion efficiency. A schematic of how biophotolysis would fit in such an ALS configuration is outlined below (Fig. 2.7). A co-generation scheme is depicted where hydrogen produced by indirect biophotolysis is sent to a fuel cell. Machinery used in composting is powered by electricity generated from the fuel cell. Water and CO2 are the main byproducts, which are sent to respective water and air revitalization subsystems for treatment.
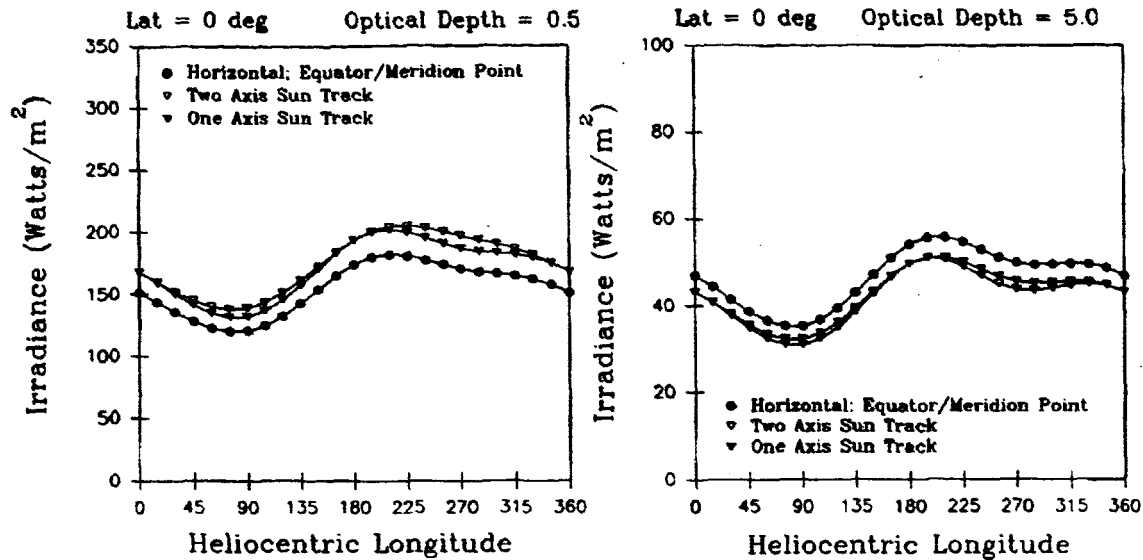
**Figure 4.6 Dust storm at the Martian equator with optical depth 0.5 – 5.0 (reproduced from (Haberle, Gwynne et al. 1993))**

## Advantages of Indirect Biophotolysis

- Can produce hydrogen at high conversion (12 H2: 1 glucose)
- Does not require oxygen
- Low energy requirements
- Operates at low temperatures and pressures
- Produces minimal microbial biomass
- Mars lighting conditions optimal for light conversion
- Mars atmospheric composition suitable for growth
- Can also degrade and produce bioplastic polymers (poly-hydroxyalkanoates) (Rijk, van der Meer et al. 2002)

## Disadvantages of Indirect Biophotolysis

- Not natural consortia
- Requires pretreatment to sugars
- Requires lighting support infrastructure
- Low efficiencies reported in literature
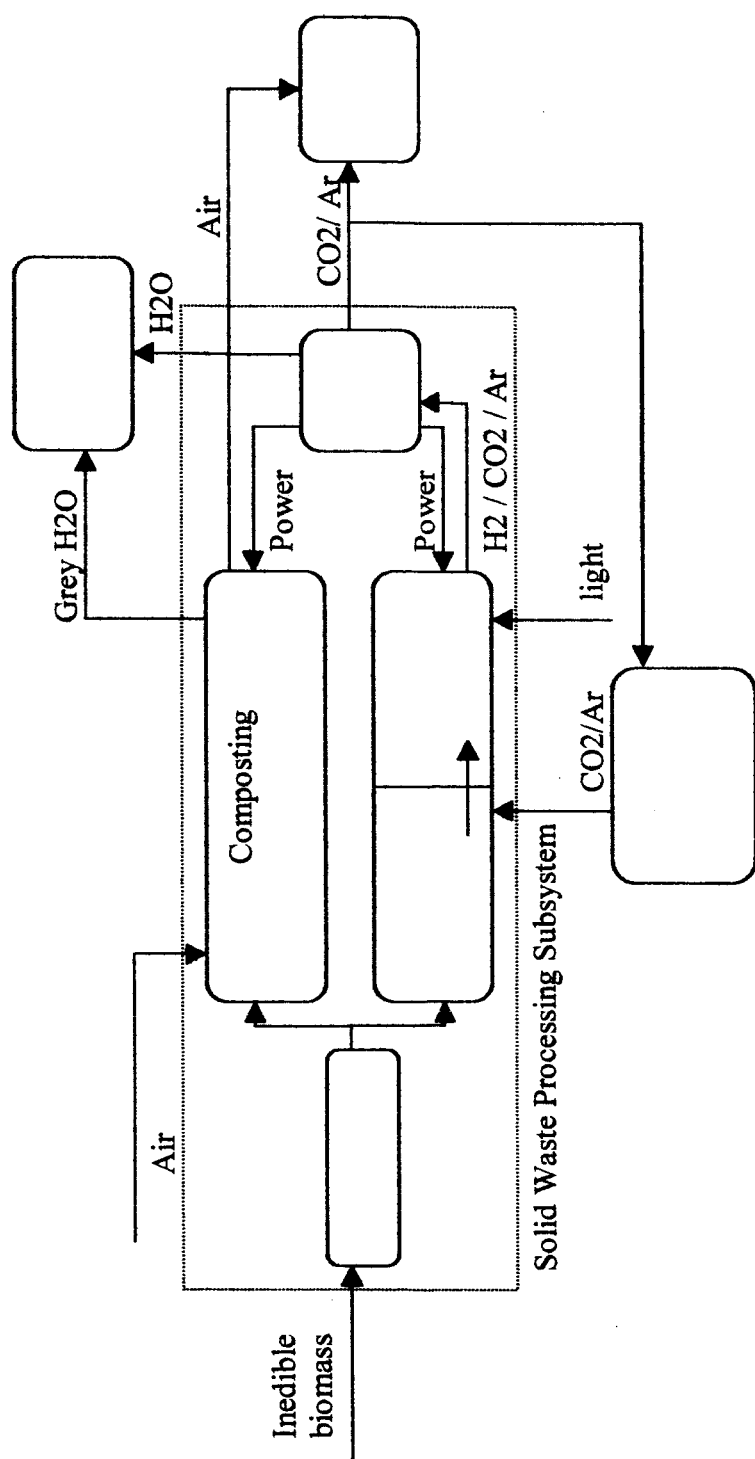- Is repressed by nitrogen atmosphere

**Figure 4.7 Composting Scheme with Indirect Biophotolysis Co-Generation**

# 5 Experiment

## 5.1 Substrate Utilization

While substrate conversion efficiencies have been widely reported in literature (see Table 3.1 below), few have reported a good characterization of each kind of substrate expected. According to Maness (2004), butyrate was found at a 2:1 ratio with acetate in the effluent from dark fermentation. This ratio can be even higher when run in batch with mixed consortia (Khanal, Chen et al. 2004). While we know that *R. sphaeroides* can metabolize these substrates, we do not know tolerance ranges and affinities for substrates or the potential for any inhibition by any of these substrates. Previous researchers have noted that butyrate is converted to hydrogen with less efficiency than other substrates, but none have done a full characterization of the butyrate sensitivity of photoheterotrophs (see Table 3.2 below). A characterization study of the metabolism of butyrate over a wide range of concentrations was conducted. An objective of this study was to fully characterize the effect on hydrogen production by butyrate and correlate this data with data points from previous literature. Several specific questions, or perhaps "proto-hypothesis" were proposed from discussions with PinChing Maness.

1. Substrate inhibition may exist at the butyrate concentrations expected in the inlet streams. This will become evident as an increasing lag phase as butyrate increases.
2. An optimal butyrate concentration for energy conversion efficiency could exist. This can be determined by comparing total hydrogen produced in the experiment.

### Table 5.1 Substrates used in hydrogen photoproduction (Sasikala, Ramana et al. 1990)

| Electron Donor | Organism | Conversion Efficiency |
|---|---|---|
| Acetate | *R. capsulatus* | 57-100 |
| Butyrate | *R. capsulatus* | 23-80 |
| Butyrate+lactate+acetate | *Rhodopseudomonas sp* | 64-70 |
| Ethanol+malate | *Rhodopseudomonas sp 7* | 48 |
| DL-Lactate | *R rubrum, R. sphaeroides, R capsulatus, R vannielii, R.sphaeroides* | 54-81, 48-78, 65-80, 78-100, 57-100 |
| DL-Malate | *R. sphaeroides* | 57-100 |
| Pyruvate | *R. palustris 42 OL* | 52 |
| Succinate | *R. capsulatus Z1* | 72 |

# Table 5.2 Various Butyrate Results Reported

| Strain | Concentration (mM) | Rate Hydrogen Produced | Total Hydrogen | Conversion Efficiency | Conditions | Reference |
|---|---|---|---|---|---|---|
| *Rhodopseudomonas sp. TN3* | Butyrate 30 mM | 30 ul/hr/mg cdw | n.r. | n.r. | 5 mM L-glutamate, 700 mL at 10000 lux | (Kim, Ito et al. 1981) |
| *Rhodopseudomonas sp. TN3* | Butyrate 30 mM + bicarbonate 10 mM | 60 ul/hr/ mg cdw | n.r. | n.r. | 5 mM L-glutamate, 700 mL at 10000 lux | (Kim, Ito et al. 1981) |
| *Rhodopseudomonas sp.* | Butyrate 24 mM | 157-255 ul/hr/cm^2 | n.r. | 54-67 | 10 mM ammonium 0.2% bicarbonate, 0.1% yeast extract, immobilized cells in 12 mL at 1 klux | (Mao, Miyake et al. 1986) |
| *Rhodobacter sp.* | Butyrate 24 mM | 185-321 ul/hr/cm^2 | n.r. | 44-67 | 10 mM ammonium 0.2% bicarbonate, 0.1% yeast extract, immobilized cells in 12 mL at 1 klux | (Mao, Miyake et al. 1986) |
| *R. rubrum G-9 BM* | 0.2% Butyrate | 14.31 ul/hr/ mg cdw | n.r. | n.r. | 10 mM ammonium immobilized cells in 5 mL at 2 klux | (Hirayama, Uya et al. 1986) |
| *Rhodopseudomonas sp. RV* | Butyrate 50 mM | 57 ul/hr/cm^2 | n.r. | n.r. | 10 mM Ammonium, 0.1% yeast extract, at 1 klux | (Miyake, Mao et al. 1984) |
| *Rhodopseudomonas sp. RV* | Butyrate 46.2 mM | 205 ul/hr/cm^2 604 ml | | 75.1 | 10 mM Ammonium, 0.1% yeast extract, immobilized cells at 1 klux | (Miyake, Mao et al. 1984) |
| *Rhodopseudomonas sp.* | Butyrate 27 mM | 7.2 ml/l/hr | 100 ml | 8.4 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |
| *R. palustris* | Butyrate 27 mM | 0 ml/l/hr | 0 ml | 0 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |
| *"Microbiology Strain"* | Butyrate 27 mM | 0.2 ml/l/hr | 4 ml | 0.3 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |
| *Rhodopseudomonas capsulata* ATCC 237B2 | Butyrate 7 mM | 32.9 ml /hr/L reactor | 23.44 mM | 50.41 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |

**Table 3.2 Continued**

| Strain | Concentration (mM) | Rate Hydrogen Produced | Total Hydrogen | Conversion Efficiency | Conditions | Reference |
|---|---|---|---|---|---|---|
| *Rhodopseudomonas capsulata* ATCC 17013 | Butyrate 7 mM | 30.8 ml /hr/L reactor | 25.57 mM | 37.6 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas capsulata* ATCC 17020 | Butyrate 7 mM | 21.5 ml /hr/L reactor | 8.34 mM | 46.59 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas capsulata* DSM 152 | Butyrate 7 mM | 30.0 ml /hr/L reactor | 20.33 mM | 58.09 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas capsulata* DSM 156 | Butyrate 7 mM | 25.4 ml /hr/L reactor | 24.53 mM | 45.85 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas capsulata* DSM 157t2 | Butyrate 7 mM | 25.8 ml /hr/L reactor | 22.25 mM | 55.76 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas capsulata* NCIB 8254 | Butyrate 7 mM | 27.1 ml /hr/L reactor | 23.01 mM | 51.36 | 0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO2 | (Stevens, Van Der Sypt et al. 1983) (Weaver, Wall et al. 1975) |
| *Rhodopseudomonas sp. RV* | Acetate 16.3 mM | 37 ul/hr/cm^2 | 117 ml | 40.2 | 10 mM Ammonium, 0.1% yeast extract, immobilized cells at 1 klux | (Miyake, Mao et al. 1984) |
| *Rhodopseudomonas sp.* | Acetate 22 mM | 25.2 ml/l/hr | 269 ml | 72.8 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |
| *R. palustris* | Acetate 22 mM | 2.2 ml/l/hr | 56 ml | 14.8 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |
| *"Microbiology Strain"* | Acetate 22 mM | 2.6 ml/l/hr | 134 ml | 35.3 | 0.8 mM Na Glutamate, 480-680 umol / m^2 / sec | (Barbosa, Rocha et al. 2001) |

## 5.2 Methods

*Rhodobacter sphaeroides SCJ* was isolated by Weaver(Weaver, Wall et al. 1975) from Jamaican soil and is currently used in hydrogen studies at the National Renewable Energy Laboratories (NREL) and was graciously donated by PinChing Maness from NREL (Maness 2004). *R. sphaeroides SCJ* was selected since it is a new strain of a well studied organism.

Media was adapted from NREL's M-1 Basal media and consisted of the following: 0.5 mM MgSO4*7H20, 0.5 mM CaCl2*2H20, 42 μM FeSO4*7H20, 60 μM EDTA, 45 μM H3BO3, 9μM MnSO4*H2O, 3 μM Na2MoO4*2H2O, 0.8 μM ZnSO4*7H2O, 0.2 μM Cu(NO3)2*3H2O, 3 μM CoCl2*6H2O, 3 μM NiCl2*6H2O, 3 μM thiamine HCl, 0.06 μM biotin, 8 μM nicotinic acid, 0.01 μM B12, 0.7 μM p-aminobenzoic acid, 4.4 mM KH2PO4, 5.2 mM K2HPO4.

Incubation media included basal media with 50 mM sodium acetate, 10 mM NH4Cl, and 1 % (by wt) of NaHCO3. Cells were incubated from plate stock in 15 mL anaerobic sterile test tubes under light for two days.

Experiments were conducted using sodium butyrate, 8 mM sodium glutamate, 0.5 % (by wt) NaHCO3, 28.8 mM KH2PO4, 34 mM K2HPO4,

Experimental Setup: 300 mL samples were incubated in 2 L capacity water-jacketed reactors held at 32 C (See Fig 3.1 below). Illumination for each reactor was provided with 65 W incandescent light bulbs (held at 12") separated by black partitions. Reactors contained 2 ports, a septum port and a gas displacement port. The gas displacement line evacuated submerged inverted graduated cylinders and contained a y-valve with a syringe sample port. Glassware was cleaned with deionized water and ethanol, and then autoclaved. Media and inoculum was added and the chamber purged with Argon before sealing. Trials were run in triplicate for butyrate concentrations ranging from 10 mM to 100 mM Sodium Butyrate. All activities were conducted at BioServe Space Technologies in Boulder, Colorado, at an altitude of about 5344 ft and a nominal pressure of 12.2 psi.
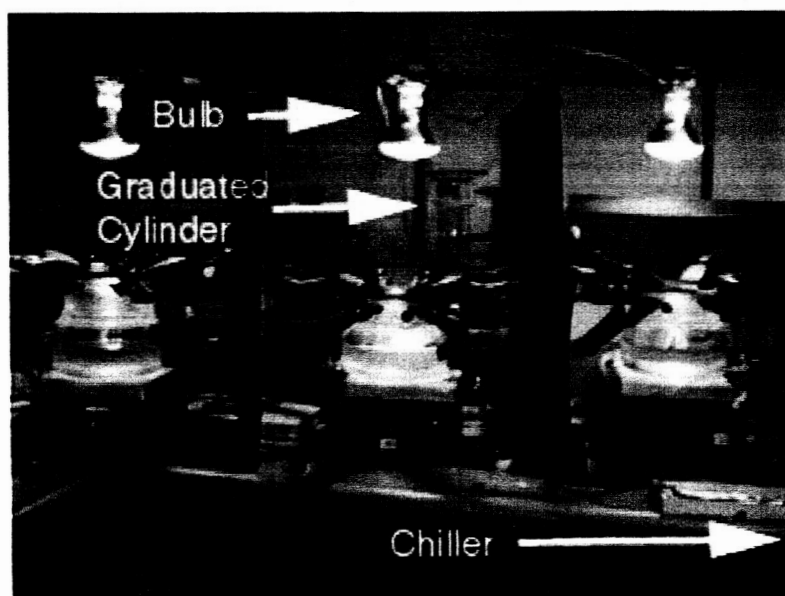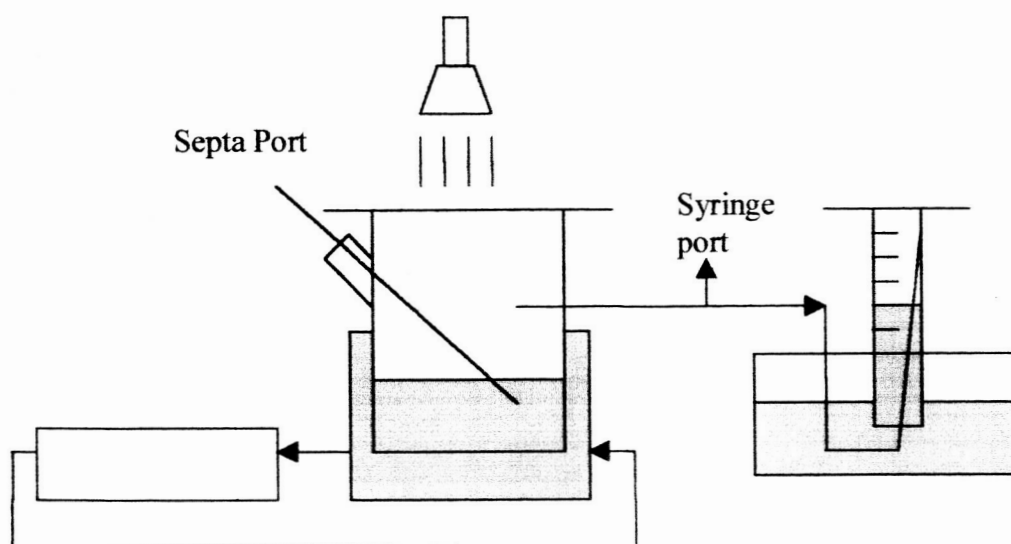
**Figure 5.1 Experimental Setup**

### 5.3 Analysis

Gas and liquid samples were taken at regular intervals. 1.0 mL of headspace from both the reactor and graduated cylinder were taken and run on an HP 5710A Gas Chromatograph with a Carboxen 1000 60/80 Mesh 15' x 1/8" SS Column at 80 C for 4 min, 32 C/ min, and 200 C for 4 minutes (See Fig. 3.2). 2.0 mL liquid samples were taken from the reactor through the septa port using 6" stainless steel cannula. pH was measured on a pH electrode. A Genesys 10 Series Spectrophotometer in absorbance mode was used to measure optical depth at 660 nm. The cells were spun and the supernatent filtered and run on a HP 1050 Series HPLC with an Aminex

HPX-87H Ion Exclusion 300 mm x 7.8 mm exclusion column with guard column and a 4mM $H_2SO_4$ mobile phase at 45 C and a flow of 0.6 L /m. A sample of 20 µL sample was injected and a UV detector was used at 210 nm to measure response. Barometric pressure was read from a 429 Nova Princo Mercury Barometer with each data point. Lastly, incandescent light bulbs were characterized with a Licor Li-1800 spectroradiometer before and after the experiment.



**Figure 5.2 HP 5710A Gas Chromatograph**

### 5.4 Data

*Hydrogen*

The volume of hydrogen was determined by multiplying the volume of the reactor and the volume of gas collected at each data point by their respective concentrations determined by gas chromatography. Using the ideal gas law (PV=nRT), where pressure is the difference of the ambient pressure with the hydrostatic water column pressure, and temperature is the ambient temperature. After the moles of hydrogen were determined they were multiplied by 22.4 L/mol to determine volume at STP conditions.

*Cell Dry Weight*

Cells were grown in the water-jacketed reactors. 20 ml were extracted, diluted, spun, washed, and the supernatent extracted. After drying, the cell dry mass was measured. Optical depth was linearly correlated to dried cell mass and used to measure mg/ml cell mass density.

*Max total hydrogen and Max rate hydrogen*

These were defined as the maximal total hydrogen produced and the maximal rate observed for any data point.

*Lag Phase Determination*

Lag phase was calculated by curve fitting cell dry weight data. Then an exponential growth tangent was drawn. The lag phase was then found by intersecting that line with a line representing the initial concentration (See Fig 3.3 below).
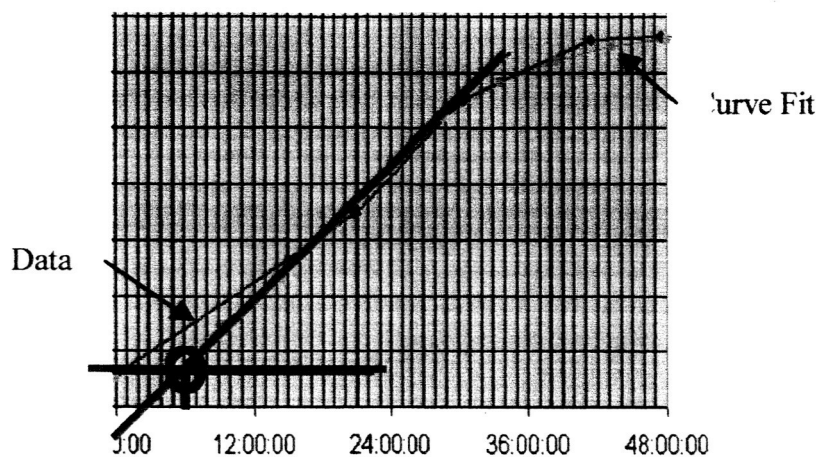


**Figure 5.3 Lag Phase Determination**

*Light Spectrum Usage*

The incandescent light profile was reported as $umol/m^2/s/nm$ (See Fig. 3.4 below). These values are typically integrated over the entire spectrum recorded, the Photosynthetically Active Range (PAR) for plants (400-700 nm), or the PAR for purple bacteria (400-950 nm) to determine the total light irradiance (Akkerman 2002). For this experiment the PAR for plants seemed unsuitable; and the PAR for purple bacteria and the total spectrum were very similar due to the geometry of the light profile (See Fig 3.4). Therefore, the total integral was taken for simplicity. The light profile was taken of each bulb before and after all the experiments. The before and after values were then averaged and then used to calculate light efficiency.
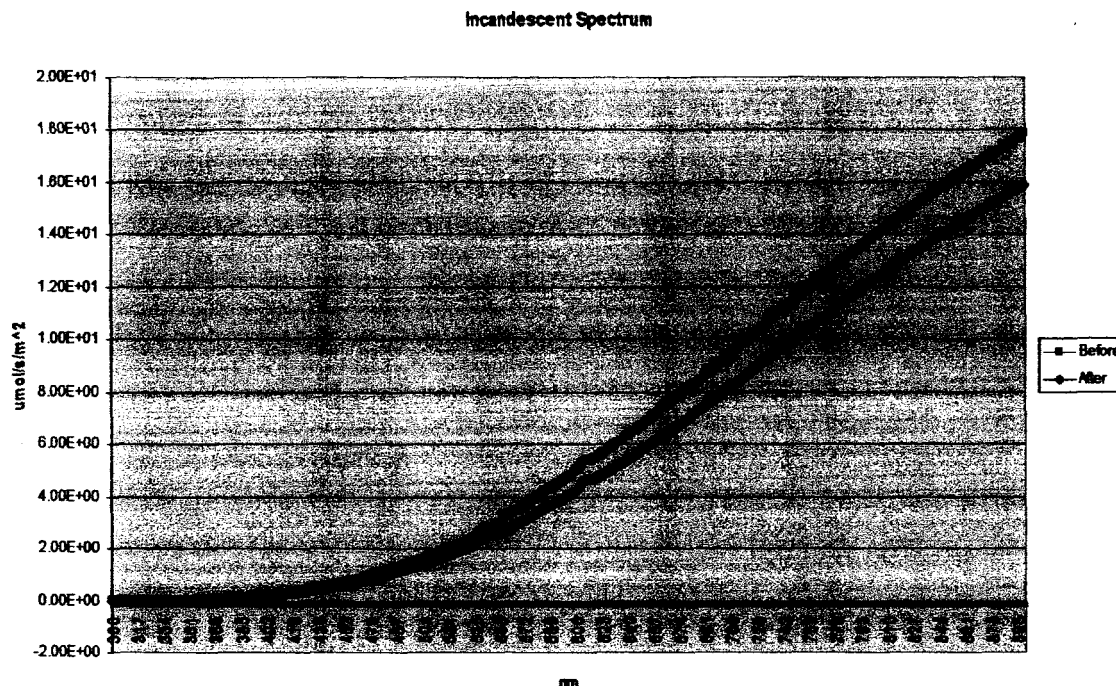
**Incandescent Spectrum**

**Figure 5.4 Light Profile of 65 W Incandescent Bulb used in the experiment**

Light efficiency was calculated with dG= 203 kJ/mol free energy.

*Carbon Nitrogen Ratio*

C/N Ratio was calculated as the average butyrate consumed per run divided by the concentration of the nitrogen compound.

# 6 Results

## 6.1 Characterization Results

Fig. 4.1- 4.3 Show raw data for cell dry weight, hydrogen produced, and butyrate consumed, respectively. Data points were run in triplicate and the values averaged. For cell dry mass (Fig. 4.1), we see that as butyrate concentration increases, the maximum growth increased until about 1.3 mg/ml density. Acetate did not reach that point, possibly due to it being the limiting substrate. Also, total hydrogen increases up to 25 mM and then decreases (Fig. 4.2). No hydrogen was reported for 100 mM butyrate. However, cell growth did occur at such concentrations. Butyrate levels fell consistently over time, indicating reproducibility (Fig. 4.3). Butyrate C/N ratio was 20-25 based on the 50 mM and 100 mM data point. Of note is the fact that actual chemostat concentration will likely be below study range.

## 6.2 Comparison of Characterization Values with Previous Literature

Butyrate conversion efficiency was calculated for all data points except 25 mM Butyrate, and is shown in Fig. 4.7. Conversion efficiency dropped from 8% to zero over the range for Butyrate and 20% for Acetate. Sasikala et al. (1990) reports efficiencies from 23-80% for butyrate and Stevens et al. (1983) observes 45-57%. Observed efficiencies were much lower than expected. This could be due to the fact that growing assimilating cells were used to produce hydrogen. Typical light efficiencies seen by Akkerman et al. (2002) shows that light efficiencies can range from 0.1-11.3%. The DOE R&D goal for 2005 is to produce hydrogen at a light conversion efficiency of 0.5% (EERE 2004). The observed light conversion efficiency (shown in Fig. 4.8 below) was from 0-0.89%, which lies in the range of expected values.

Typical Max H2 Reported from literature is 30-60 ul/mg/hr as reported by Kim et al. (1981). 0-7.7 umol/mg/hr (0-173 ul/mg/hr) was found in this experiment (See Fig 4.6). This is about the same magnitude as in reported literature.

## 6.3 Substrate Inhibition Results

An increasing trend in increasing lag phase was observed (Fig. 4.4). A Student's 1 tail unpaired t-test was performed on butyrate curves with 100 mM. The differences in the far data points (10, 17 mM) was shown to be significant, but the other data points were not. Additionally, a Student's t-test was used to compare 25 mM Acetate to 25 mM Butyrate, and was significant. Since lag phase increases with substrate concentration, substrate inhibition correlates with butyrate concentration.
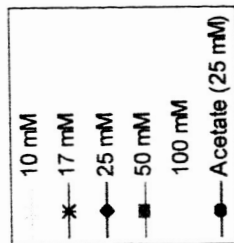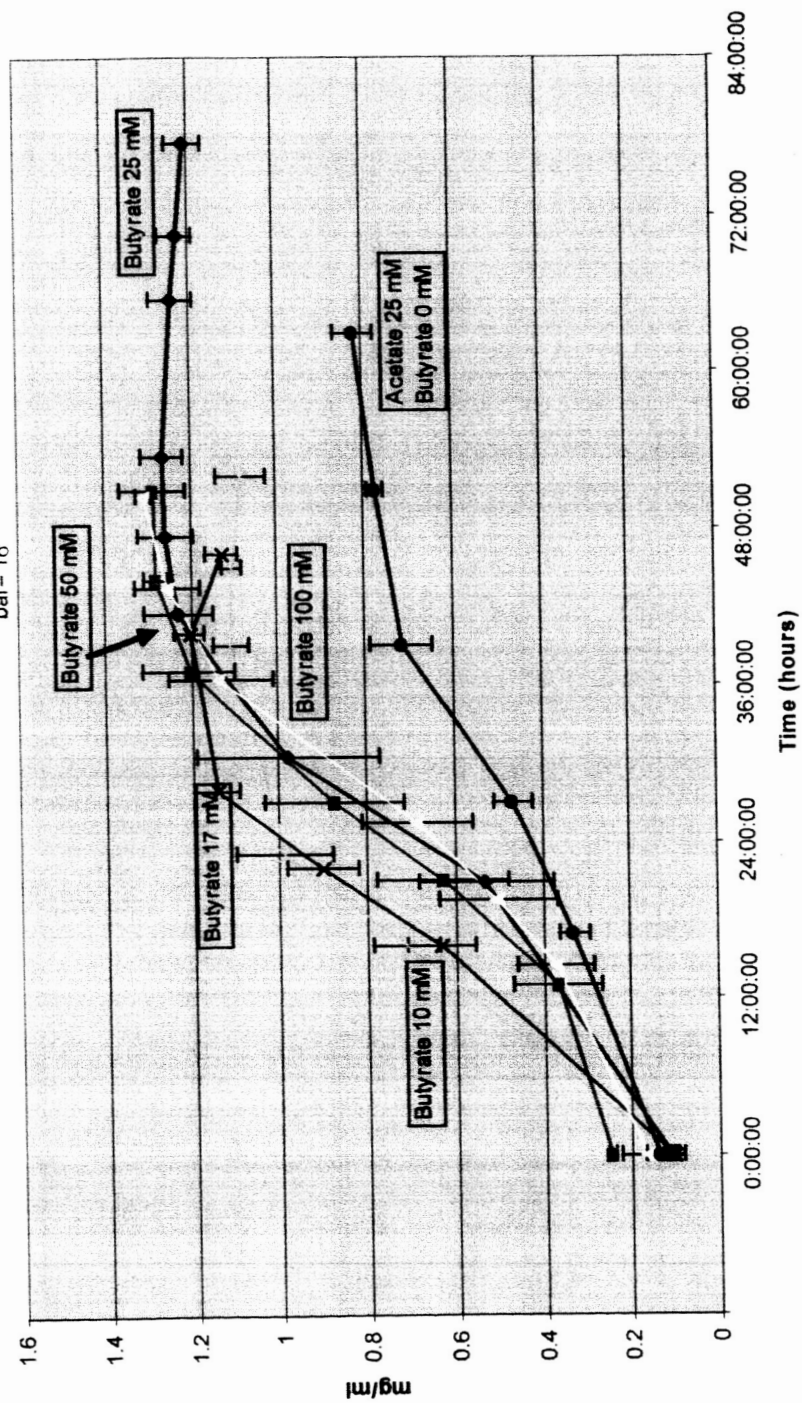
## 6.4 Optimal Concentration

Both maximal total hydrogen and maximal hydrogen production rate occurred at 25 mM Butyrate (See 4.5 and 4.6). 25 mM Acetate was run as a comparison and did not perform as well as butyrate at that same concentration.

Fig 4.1 Cdw vs time

N=3

bar= 1σ

Legend:
- 10 mM
- 17 mM
- 25 mM
- 50 mM
- 100 mM
- Acetate (25 mM)

y-axis: mg/ml

x-axis: Time (hours)

Labels on plot:
- Butyrate 25 mM
- Butyrate 50 mM
- Butyrate 100 mM
- Butyrate 17 mM
- Butyrate 10 mM
- Acetate 25 mM Butyrate 0 mM

Fig 4.2 H2 vs time
N=3
bar= 1σ

mol H2

0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005

0:00:00   12:00:00   24:00:00   36:00:00   48:00:00   60:00:00   72:00:00   84:00:00

time (hours)

Butyrate 25 mM

Acetate 25 mM
Butyrate 0 mM

Butyrate 17 mM
Butyrate 50 mM
Butyrate 10 mM

10 mM
17 mM
25 mM
50 mM
100 mM
Acetate (25 mM)

Fig 4.3 Butyrate vs time

N=3

bar= 1σ

Legend:
- 10 mM
- 17 mM
- 50 mM
- 100 mM
- Acetate 25 mM

mM Butyrate

hour

Butyrate 100 mM

Butyrate 50 mM

Acetate 25 mM
Butyrate 0 mM

Butyrate 17 mM

Butyrate 10 mM
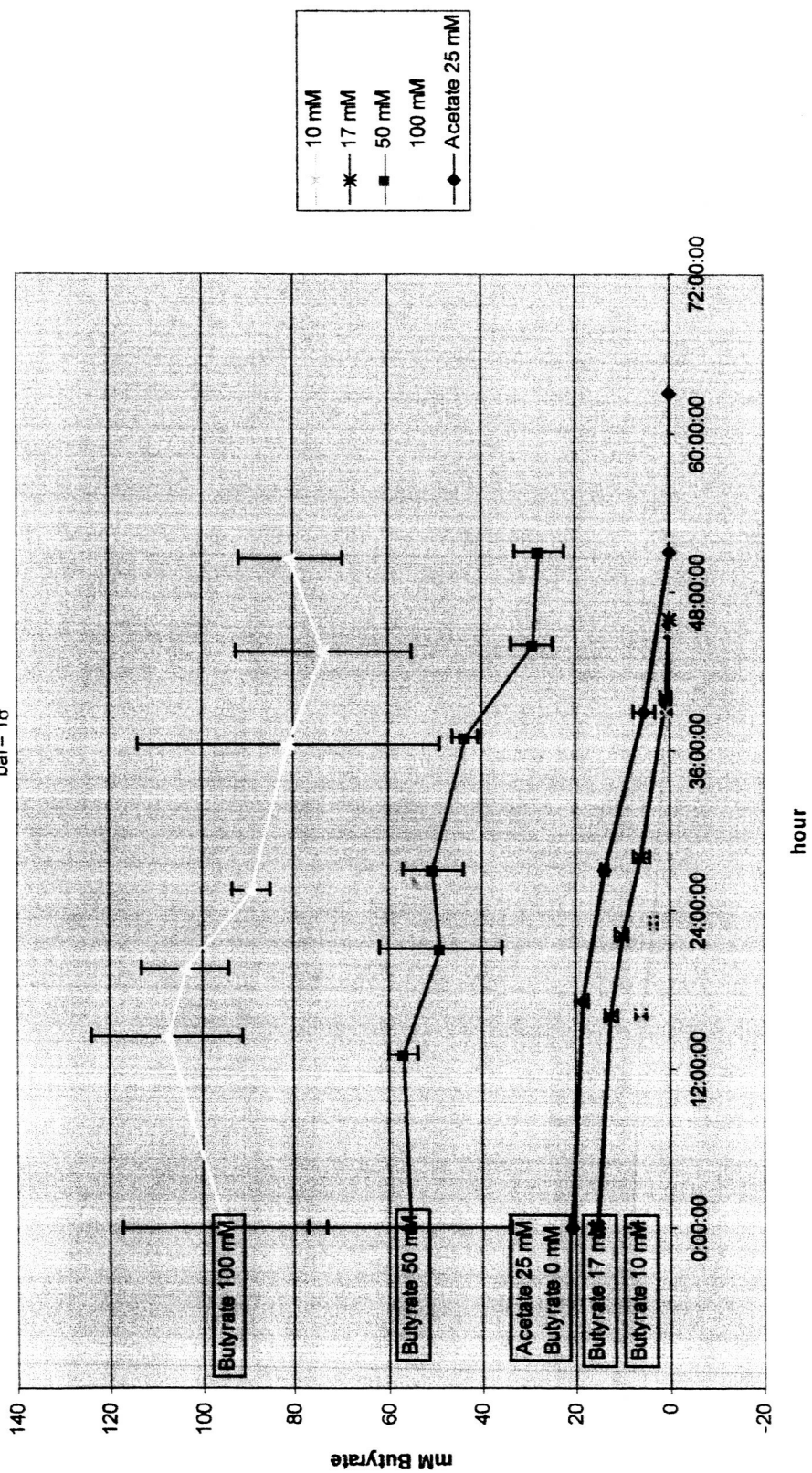
# Fig 4.4 Butyrate Concentration vs Lag Phase



Legend: Butyrate, Acetate

Data labels on chart:
- N=3, σ=0.58, x̄=2.66, p=0.011*
- N=3, σ=0.5, x̄=1.5, p=0.038*
- N=3, σ=2.2, x̄=6.5, p=0.089*
- N=3, σ=2.0, x̄=2.8, p=0.050+
- N=3, σ=6.1, x̄=8.5, p=0.42*
- N=3, σ=2.0, x̄=9.3, p=0

Axis labels:
- Lag Phase (hr): 0, 2, 4, 6, 8, 10, 12, 14, 16
- Concentration (mM): 10, 17, 25, 50, 100
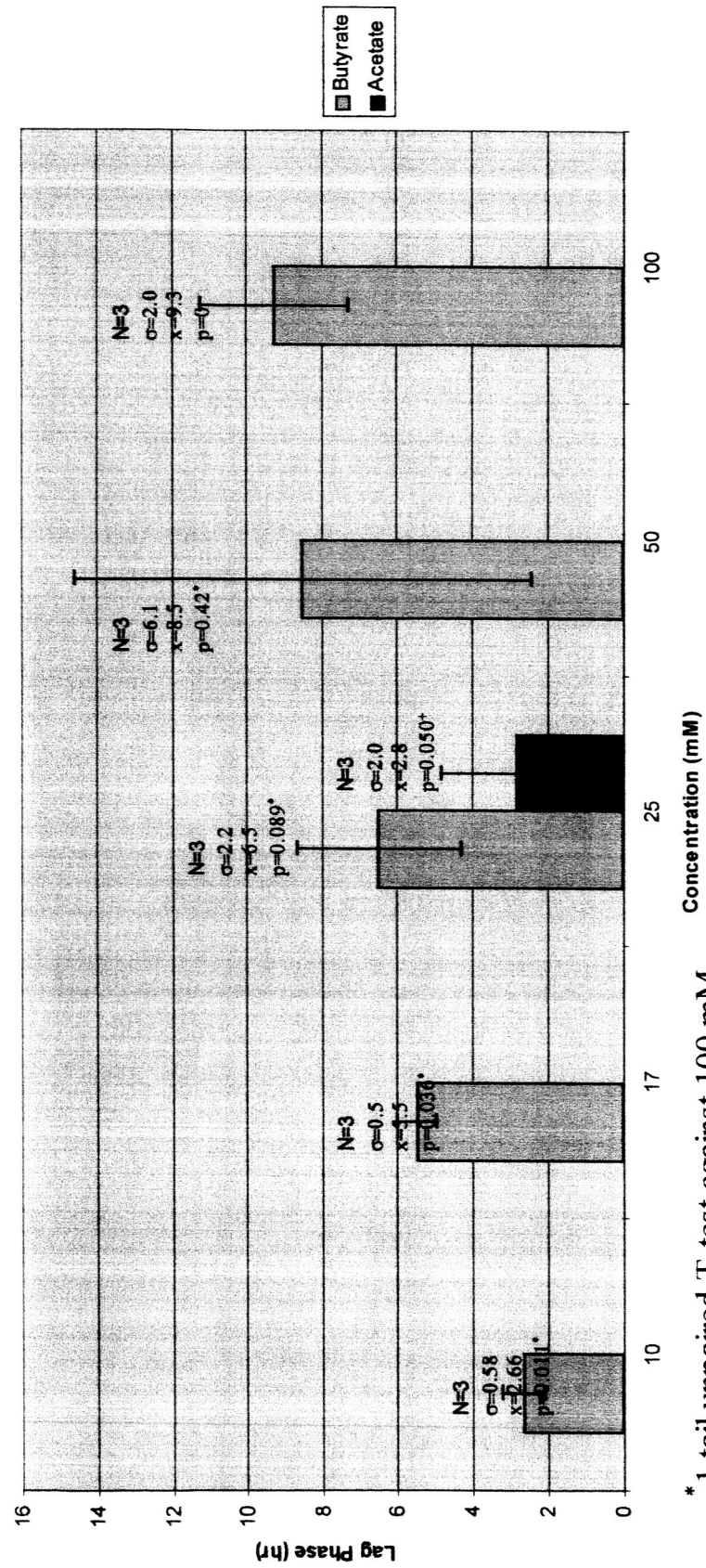
* 1 tail unpaired T-test against 100 mM

+ 1 tail unpaired T-test against 25 mM Butyrate

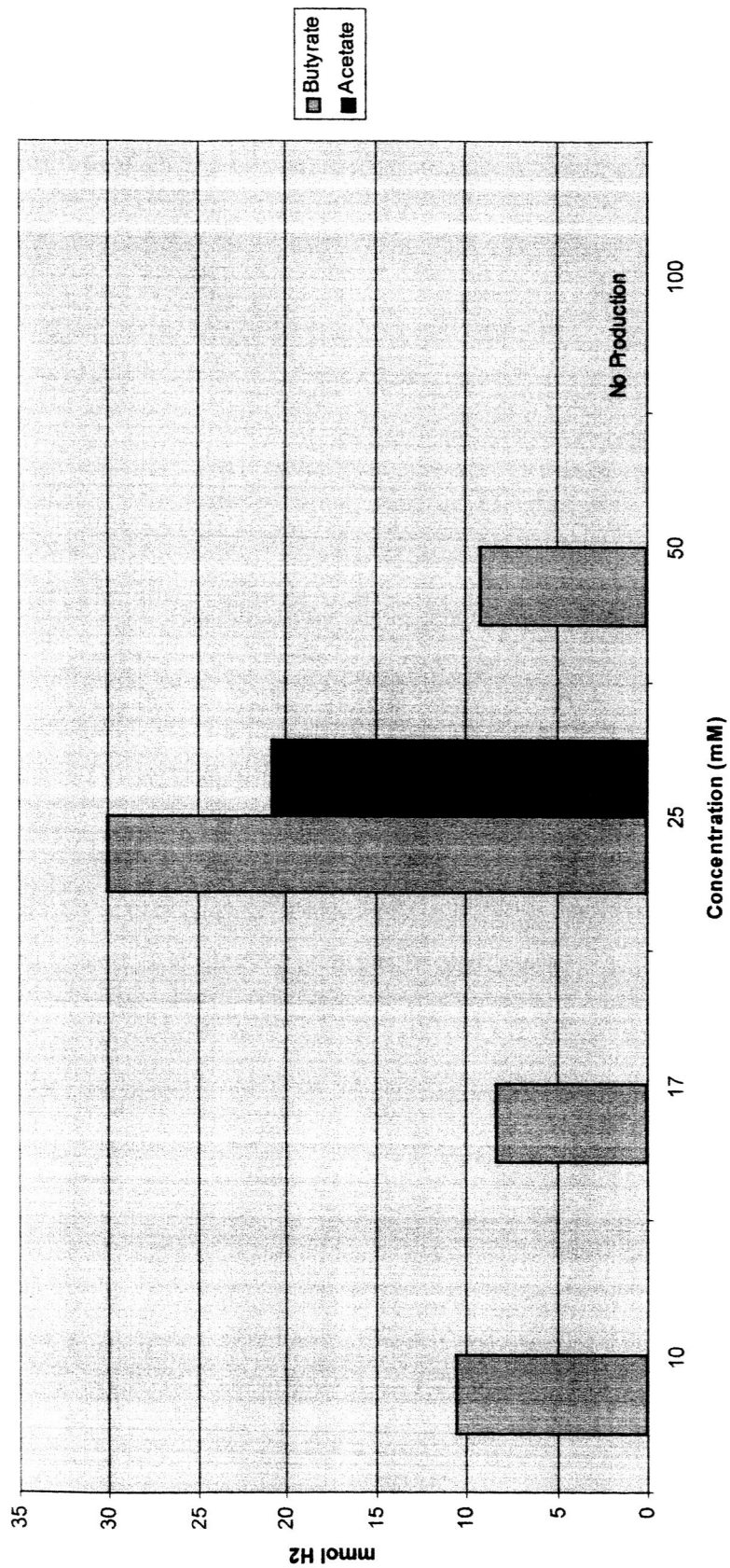Fig. 4.5 Butyrate Concentration vs Max Hydrogen Production

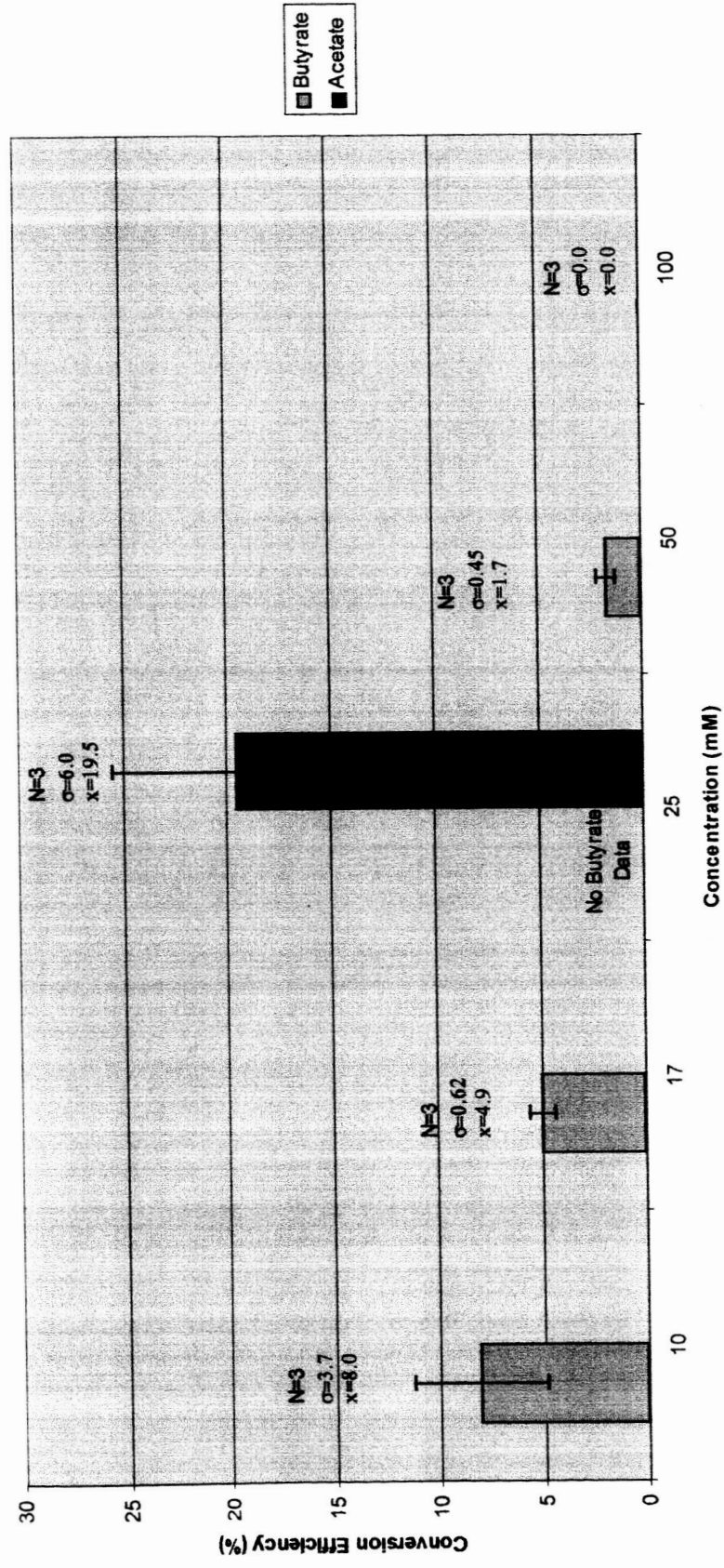Fig. 4.7 Substrate Efficiency vs Concentration
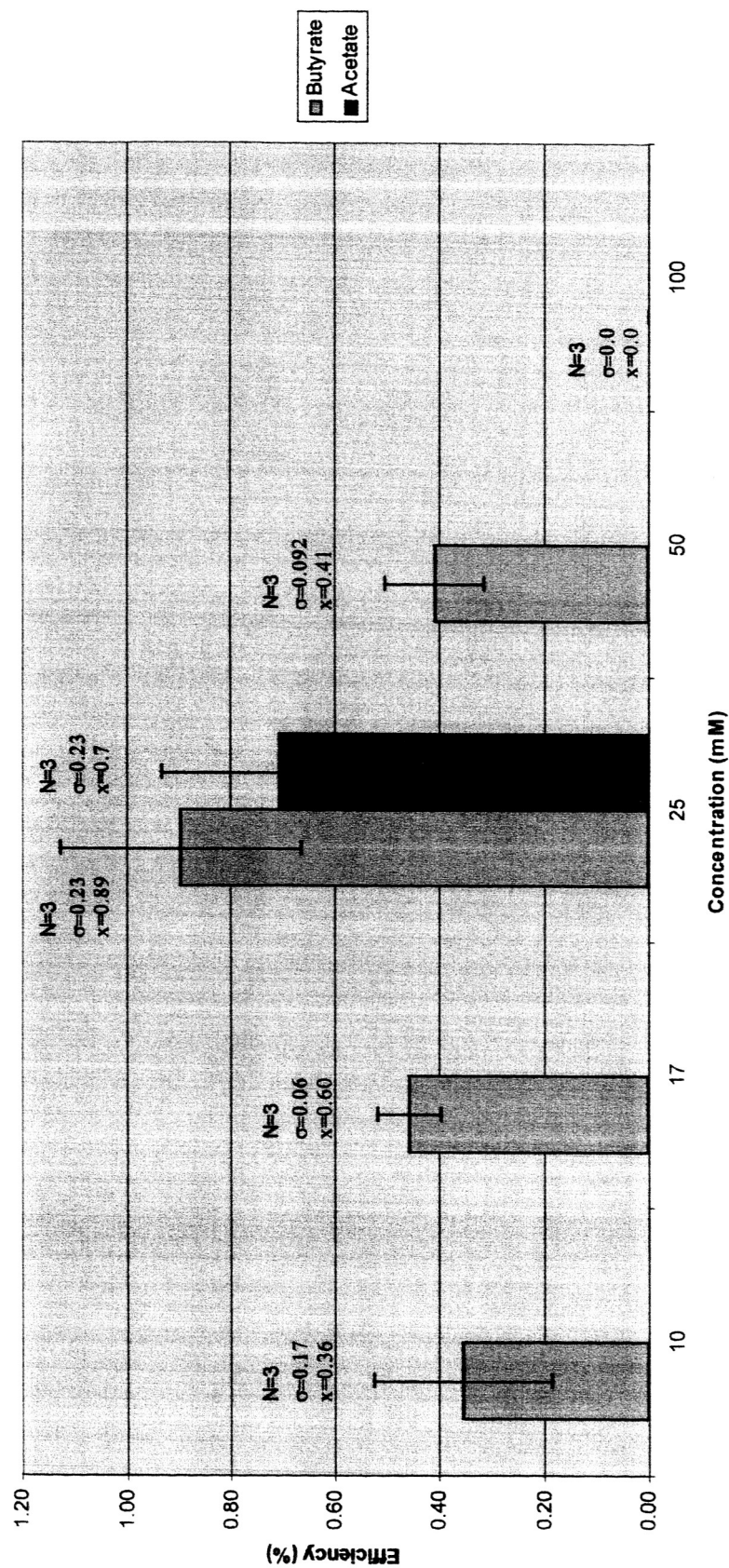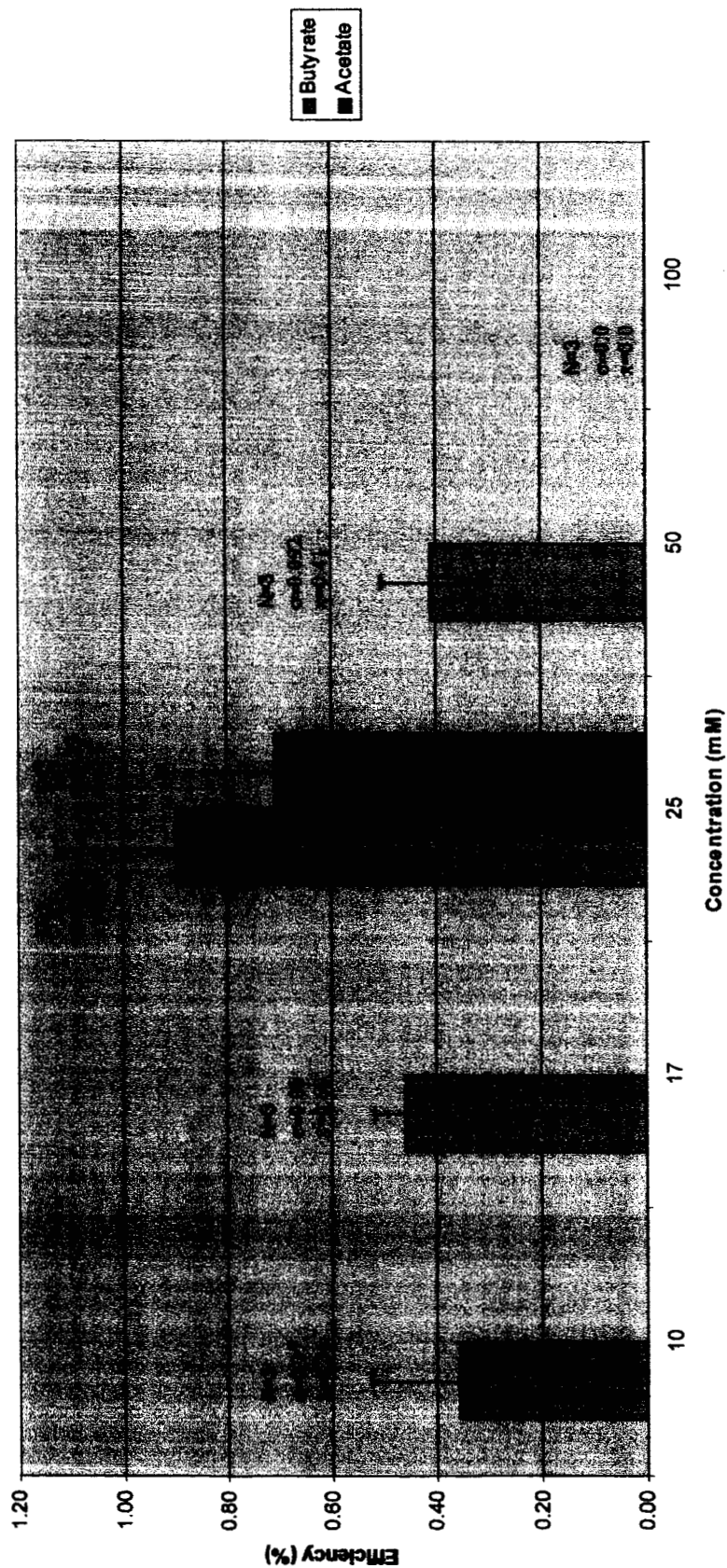
Fig. 4.8 Light Efficiency vs Concentration

Fig. 4.8 Light Efficiency vs Concentration

# 7 Conclusion

Indirect biophotolysis has the potential to allow energy co-generation on Mars while at the same time degrading and recycling waste biomass to carbon dioxide. Mars application of this process has not been extensively explored. There are several potential advantages of using indirect biophotolysis. The production of H2, which is a useful byproduct from a waste reduction stream, is achieved at potentially greater efficiency than electrolysis. Incident light levels may have presented some concern for the viability of using a system such as this. Interestingly however, the light PAR and intensity on the surface of Mars is actually in an ideal range for this process. Finally, in addition to its application to a Mars base life support system, biological hydrogen production is a growing area of recent interest in terms of alternative terrestrial renewable energy production. While some dynamic parameters are still unclear, much work is being done to characterize fully the system described previously. In this study, the hydrogen production response to butyrate was characterized. Based on this data, optimal hydrogen production was found at 25 mM Butyrate, falling off and ceasing by 100 mM. Butyrate outperformed acetate at that concentration. Conversion and light efficiency are somewhat lower than reported in literature, but similar production rates are observed. Furthermore, a correlation between lag phase and butyrate is suggested, possibly indicating substrate inhibition.

# 8 Employment and Future Plans

I have elected to not reapply for a continuation of the GSRP grant. I have decided to take some time off from research to join the Peace Corps for two years. I will be doing water and sanitation engineering for developing areas in the Philippines. This is not a total step back from my work; much of what I will be doing in the Peace Corps will cross-train me in other areas of environmental engineering. In the future, I will return to finish my PhD in environmental engineering. I hope to establish a career in the research area of microbial engineering in space for manned spaceflight applications.

# 9 Papers, Presentations, Awards

Lee, Jackson Z, and D. Klaus, P. Maness, J. Spear. "Characterization of the Effect of Butyrate on Hydrogen Production in Biophotolysis for use in Martian Resource Recovery" *Int. J. of Hydrogen Energy* (Proposed Submission)

Guest Researcher, Biodegradable Polymer Group AIST, East Asia Pacific Summer Institutes Participant, Tsukuba-shi, Japan

Beverly Sears Graduate Student Award, University of Colorado

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