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Effects of Mars Regolith Analogs, UVC radiation, Temperature, Pressure, and pH on the Growth and Survivability of Methanogenic Archaea and Stable Carbon Isotope Fractionation: Implications for Surface and Subsurface Life on Mars

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Effects of Mars Regolith Analogs, UVC radiation, Temperature, Pressure, and pH on the Growth and Survivability of Methanogenic Archaea and Stable Carbon Isotope Fractionation: Implications for Surface and Subsurface Life on Mars

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Abstract

Mars is one of the suitable bodies in our solar system that can accommodate extraterrestrial life. The detection of plumes of methane in the Martian atmosphere, geochemical evidence, indication of flow of intermittent liquid water on the Martian surface, and geomorphologies of Mars have bolstered the plausibility of finding extant or evidence of extinct life on its surface and/or subsurface. However, contemporary Mars has been considered as an inhospitable planet for several reasons, such as low atmospheric surface pressure, low surface temperature, and intense DNA damaging radiation. Despite the hostile conditions of Mars, a few strains of methanogenic archaea have shown survivability in limited surface and subsurface conditions of Mars. Methanogens, which are chemolithoautotrophic non-photosynthetic anaerobic archaea, have been considered ideal models for possible Martian life forms for a long time. The search for biosignatures in the Martian atmosphere and possibility of life on the Martian surface under UVC radiation and deep subsurface under high pressure, temperature, and various pHs are the motivations of this research. Analogous to Earth, Martian atmospheric methane could be biological in origin. Chapter 1 provides relevant information about Mars' habitability, methane on Mars, and different strains of methanogens used in this study. Chapter 2 describes the interpretation of the carbon isotopic data of biogenic methane produced by methanogens grown on various Mars analogs and the results provide clues to determine ambiguous sources of methane on Mars. Chapter 3 illustrates the sensitivity of hydrated and desiccated cultures of halophilic and non-halophilic methanogens to DNA-damaging ultraviolet radiations, and the results imply that UVC radiation may not be an enormous constraint for methanogenic life forms on the surface of Mars. Chapters 4, 5, and 6 discuss the data for the survivability, growth, and morphology of methanogens in presumed deep subsurface physicochemical conditions such as temperature, pressure, hydrogen concentration, and pH of Mars. Finally, chapter 7 provides conclusions, limitations of

the experiments, and future perspective of the work. Overall, the quantitative measurements obtained in the various sections of this novel work provide insights to atmospheric biosignatures and survivability of methanogenic organisms on the surface and subsurface of Mars.

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Dedication

This work is dedicated to my loving husband Bachan Kumar Sinha, who supported and guided me from the start to the end of my PhD program.

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2. **Sinha, N**., and T. A. Kral, 2016, Effect of UVC radiation on hydrated and desiccated cultures of halophilic and non-halophilic methanogenic archaea: Implications for life on the surface of Mars (Under review) **[Chapter 3]**

3. **Sinha, N.**, S. Nepal, T. A. Kral, P. Kumar, 2016, Effects of Temperatures and High Pressures on the Growth and Survivability of Methanogens and Stable Carbon Isotope Fractionation: Implications for Deep Subsurface Life on Mars: Origins of Life and Evolution of Biospheres (Under review) **[Chapter 5]**

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Chapter 1

Introduction

1.1 Mars

1.1.1 An overview

"Is there any life out there?" To answer this question, we have been endeavoring for a long time and searching for life on other celestial bodies by different modes of operations such as ground based telescopes, flybys, orbiters, landers, and rovers. Many successful and unsuccessful missions have been deployed since the early sixties to study the atmospheres, surfaces and subsurface conditions of extraterrestrial bodies. Mars, which is the fourth planet in our Solar System, is the most extensively studied terrestrial planet. One reason is that Mars is the second closest planet to Earth after Venus. Additionally, Earth and Mars were formed about the same time, around 4 billion years ago, and they may have similar physical and chemical environments during that time. Over the course of its evolution, Mars cooled faster because of its smaller size. Eventually Mars became colder (210 K [−63 °C]) and drier than Earth. The diameter of Mars is about 6,792 km, which is about half of the diameter of Earth. Mars has no magnetic field or tectonic plates. Its surface is continuously bombarded with high energy solar and galactic cosmic rays (Pavlov et al., 2012). The atmosphere of Mars is mainly composed of carbon dioxide (95.3%) followed by nitrogen (2.7%), argon (1.6%), oxygen (0.13%), carbon monoxide (0.08%), and some trace gases such as methane. More than half of Mars has the average surface pressure of 6.1 mbar, which is below the triple-point vapor pressure of water. (Carr and Head, 2010; Haberle et al., 2001; Malin and Edgett, 2000). Therefore, water cannot exist in liquid form on the surface with the present temperature and pressure conditions of Mars (Haberle et al., 2001) except under certain conditions (see below; Martín-Torres et al., 2015). If liquid water forms due

to increasing temperature, water ice sublimates becoming either solid or gaseous making it a dry planet. Several factors such as extremely low temperature, low pressure, lack of liquid water, and intense cosmic radiation make Mars an inhospitable planet. However, geomorphological and geochemical evidence (Head et al., 2008; Malin and Edgett, 2000) points to the fact that Mars was warmer and wetter in the past. It had a thick atmosphere and was protected from intense damaging radiation. Apart from the hostile conditions of present day Mars, it is one of the suitable candidates where we could search for possible extinct or extant life on its surface, near surface or deep subsurface. The reasons are discussed in the following sections.

1.1.2 Habitability of Mars

In 1993, NASA began the "Mars Exploration Program" (MEP) to explore the climate, geology, habitability potential, and the possibility to send humans to Mars. Initially, NASA's MEP had the strategy "follow the water". As we know, water is an essential key to life. On Earth, where there is water, we have found life. Earlier Mars missions such as the 2001 Mars Odyssey, Mars Exploration Rovers, Mars Reconnaissance Orbiter, and Mars Phoenix Lander were designed to "follow the water". After intensive research and evidence of surface and subsurface water ice on Mars (Carr and Head, 2010; Feldman et al., 2002; Head et al., 2008), NASA's MEP is currently following the exploration strategy of "Seek Signs of Life" such as searching for chemical biosignatures in soil, rocks or gas.

In a recent study, Ojha et al. (2015) presented evidence of episodic liquid water on Mars. This recent discovery is based on evidence of the occurrence of dark streaks called "recurring slope lineae" (RSL) throughout the planet, mainly in the deep canyon regions, which may be the results of intermittent activity of water. These RSLs were observed in the images taken by Mars

Reconnaissance Orbiter's (MRO), High Resolution Imaging Science Experiment (HiRISE), and the mineral-mapping instrument of MRO's Compact Reconnaissance Imaging Spectrometer for Mars (CRISM). The spectral signatures suggest the presence of hydrated salt on the downhill steep slope of the RSL. The hydrated salt most likely consists of magnesium chlorate, magnesium perchlorate, and sodium perchlorate. Some perchlorate can decrease the freezing point of liquids as low as -70 deg C. These dark-streak RSLs darken in the warm season and fade in the colder season, which suggests the seasonal flow of water on Mars. Nevertheless, the source of water is still unknown. Whether the water is coming from the surface/subsurface of melting ice or absorption of water vapor from the Martian atmosphere is still a puzzle. However, it should be noted that this liquid water on Mars appears to be too briny to support Earth's life forms (Martin-Torres et al., 2015).

Additionally, geological evidence suggests that Mars was warmer and wetter about four billion years ago (Baker, 2001; Malin and Edgett, 2000). The stable isotopic ratios of oxygen and hydrogen in the Martian atmosphere indicate that a substantial amount of water has been lost to space (Mahaffy et al., 2013; Owen et al., 1988; Webster et al., 2013). However, mathematical models imply that a substantial amount of water still remains today from the ancient era and exists as near surface cryosphere and as ground water residing deeper in the crust (Clifford et al., 2010). A few subsurface conditions such as radiogenic heating, the pressure above the triple point of water, and the presence of dissolved salts could form the Martian ground water in the cryosphere (Clifford et al., 2010; Weiss et al., 2000). A Martian pressure-temperature model for a potential Martian biosphere suggests that liquid water could be available up to 310 km below the surface of Mars (Jones et al., 2011). Moreover, the surface features on Mars such as channels, chaotic and fretted terrains, and some rifted basins (chasmata) might be formed by ancient hydrothermal systems (Farmer, 1996).

Life needs liquid water, energy sources, carbon and many trace elements. In order to search for subsurface life on Mars, it is important to locate sources of energy and carbon in Martian subsurface environments in addition to water. Based on geochemical and thermodynamic considerations, it has been suggested that geologically derived hydrogen could be the primary energy source (Chapelle et al., 2002). The interaction of hydrothermal fluid with reduced basalt can produce H_2 through serpentinization (Lyons et al., 2005; Oze and Sharma, 2005; Schulte et al., 2006). Additionally, on Earth, hydrogen is present in the free gas phase up to 9-58% by volume and dissolved hydrogen up to 7.4 mM concentration from water-rock reactions has been reported, which could be available for a surface and/or deep subsurface biosphere (Sherwood Lollar et al., 2007). The carbon could be derived from one or more sources such as dissolved CO_2 in the ground water, diffusion of atmospheric CO_2 into the regolith (Weiss et al., 2000), and sequestration of CO_2 in carbonates (Farmer, 1996; Kral et al., 2014; Varnes et al., 2003). The geochemical models for Martian hydrothermal systems also suggest ample amounts of chemical energy may be available for putative Martian microorganisms if the composition of host rock is the same as the composition of Martian meteorites (Varnes et al., 2003). All these pieces of evidence direct us to search for extinct or extant Martian life beneath the surface of Mars. Furthermore, the discovery and the study of a subsurface biosphere on Earth generated enthusiasm for finding life in the subsurface of Mars. Interestingly, subterranean organisms outnumber life on Earth's surface (Whitman et al., 1998).

The detection of seasonal methane gas in the Martian atmosphere is another source of excitement stimulating the search for life on Mars. Methane in the Martian atmosphere was detected by the Planetary Fourier Spectrometer (PFS) on Mars Express (Formisano et al., 2004), the Fourier Transform Spectrometer (FTS) at Canada-French-Hawaii Telescope (CFHT; Krasnopolsky et al., 2004), a high-resolution spectrograph (CSHELL) at the Infrared Telescope Facility (IRTF), the Gemini telescope (Mumma et al., 2004; Mumma et al., 2009), and the tunable laser spectrometer of the Sample Analysis at Mars instrument suite onboard the Curiosity rover at Gale

crater (Webster et al., 2015). Methane on Mars is temporal, seasonal, and localized. The half-life of methane in a planetary atmosphere is about 300 years (Hunten, 1979), so in order to be detected on Mars, methane would need to be replenished continuously. The source/sources of seasonal and non-uniformly distributed methane on Mars is still uncertain, it could be biotic, abiotic or both.

Methane on Mars may be ancient or contemporary. If ancient, it may have been stored in clathrate ices and released into the atmosphere from time to time as environmental conditions changed (Chastain and Kral, 2012; Chastain and Chevrier, 2007). Various potential sources of methane in the Martian atmosphere have been suggested, such as volcanic, meteoritic, cometary, hydrogeochemical, and biogenic sources (Atreya et al., 2007). On Earth, however, about 90 to 95% of atmospheric methane has a biological origin, either from living organisms or decay of organic matter (Atreya et al., 2007). Hence, one explanation for the finding and non-uniform distribution of methane on Mars could be localized microbial sources, either extinct or extant, such as methanogens.

1.2 Methanogens

1.2.1 Methanogens: Ideal Candidates for Life on Mars

Methanogens, which belong to the domain Archaea, have been considered ideal models for possible Martian life-forms for a long time (Boston et al., 1992; Chapelle et al., 2002; Kral et al., 1998; Weiss et al., 2000). Methanogens are chemolithoautotrophic, non-photosynthetic anaerobic archaea. They produce methane as the primary end product of their metabolism. They are classified into different groups depending on the metabolic pathway utilized: a) hydrogenotrophic (use H_2 and CO_2) b) methylotrotrophic (use methyl compounds) c) acetoclastic (use acetate). Most methanogens use H_2 for their energy source and CO_2 for their carbon source.

The natural habitats of methanogens are wetlands, the digestive tract of animals (such as ruminants and humans), sewage digesters, and aquatic sediments in lakes, ponds, swamps, marshes, and rice paddies. They can also live in a wide range of extreme environments, such as from alkaline to acidic environments, from permafrost to hot springs, from freshwater to marine environments. Certain strains of methanogens can tolerate low pressure, desiccation (Kral et al., 2011), and very cold temperature (Reid et al., 2006), similar to conditions present on Mars. Also some methanogenic strains can tolerate very high-pressure (Navita et al. 2015, submitted) presumably a deep subsurface condition of Mars.

On Earth, the most dominant microbial community in hydrothermal waters is hydrogen consuming, methane-producing archaea (Chapelle et al., 2002). Analogous to Earth's subsurface biosphere microbial community, chemolithoautotrophic microorganisms would be the most appropriate models for plausible microorganisms in the subsurface of Mars (Fish and Giovanni, 1999, Mancinelli, 2000).

Considering several points such as growth and survivability of methanogens in various extreme environments and Mars simulated conditions, abundance of CO_2 in the Martian atmosphere, and detection of methane in the Martian atmosphere, methanogens have been considered ideal candidates for life on Mars (Boston et al., 1992; Chapelle et al., 2002; Chastain and Kral, 2012; Chastain and Kral, 2010a, b; Kendrick and Kral, 2006; Kral and Altheide, 2013; Kral et al., 2011; Kral et al., 2004; Kral et al., 2014; Kral et al., 1998; Kral et al., 2015; McAllister and Kral, 2006; Moran et al., 2005; Ormond and Kral, 2006; Sinha and Kral, 2015; Ulrich et al., 2010; Weiss et al., 2000).

1.2.2 Methanogens Used in this Study: An overview

The four different types of methanogenic strains used in different projects of this study are as follows:

1. *Methanothermobacter wolfeii* (OCM36; Figure 1.1)
2. *Methanosarcina barkeri* (OCM38; Figure 1. 2)
3. *Methanobacterium formicicum* (OCM55; Figure 1.3)
4. *Methanococcus maripaludis* (OCM151; Figure 1.4)

The physiological characteristics of these four methanogens are shown in Table 1.1:

The pure cultures of *M. wolfeii* (OCM36), *M. barkeri* (OCM38), *M. formicicum* (OCM55), and *M. maripaludis* (OCM151) were initially obtained from the Oregon Collection of Methanogens, Portland State University, Portland, OR. Each species was grown in its respective growth-supporting medium.

1.2.3 Preparation of Media for Methanogens

1.2.3.1 Preparation of Bicarbonate Buffer

The bicarbonate buffer was prepared by dissolving 4g of NaOH in 1L of deionized water in a plastic bottle followed by saturation with carbon dioxide gas for 30 minutes. The bottle was left open in a Coy anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen, overnight for deoxygenation. The next day the bottle of buffer was capped and ready to use in media preparation.

1.2.3.2 Preparation of Sodium Sulfide Solution

A 2.5 % Na_2S solution, which is a reducing agent, was prepared by dissolving 2.5 gm of Na_2S in 100 mL of deionized water followed by saturation with argon gas for 20 minutes in an anaerobic glass bottle. The bottle was sealed with a rubber stopper, crimped, and autoclaved for sterilization.

1.2.3.3 Preparation of Solution A, B, C, and D

The growth supporting liquid media for the four different types of methanogens contains four basic stock solutions, labeled as solution A, solution B, solution C, and solution D. These solutions are comprised of the following compounds:

Solution A

100 g/L NH_4Cl

100 g/L $MgCl_2.6H_2O$

40 g/L $CaCl_2.2H_2O$

Solution B

200 g/L $K_2HPO_4.3H_2O$

Solution C

0.5 g/L Resazurin

Solution D

500 mg/L	Na_2-EDTA.$2H_2O$	20 mg/L	$NiSO_4.6H_2O$
150 mg/L	$CoCl_2.6H_2O$	10 mg/L	H_2SeO_3
100 mg/L	$MnCl_2.4H_2O$	100 mg/L	$ZnCl_2$
100 mg/L	$FeSO_4.7H_2O$	40 mg/L	$AlCl_3.6H_2O$

30 mg/L	$Na_2WO_4.2H_2O$	10 mg/L	H_3BO_3
20 g/L	$CuCl_2.2H_2O$	10 mg/L	$Na_2MoO_4.2H_2O$

1.2.3.4 Preparation of MM, MS, MSF, and MSH media

(i) MM medium (Xun et al., 1988) for *M. wolfeii*

To prepare 100 mL of MM medium, 1 mL of solution A, 0.2 mL solution B, 0.2 mL solution C, and 0.1 mL solution D were added to a flask. Then bicarbonate buffer was added to the flask inside the anaerobic chamber to make 100 mL of MM medium. The flask was left in the anaerobic chamber overnight to de-oxygenate.

(ii) MS medium (Boone et al., 1989) for *M. barkeri*

To prepare 100 mL of MS medium, 1 mL of solution A, 0.2 mL solution B, 0.2 mL solution C, 0.1 mL solution D, 0.2 g yeast extract, 0.2 g trypticase peptone, and 0.05 g of mercaptoethanesulfonic acid were added to a flask. Then bicarbonate buffer was added to the flask inside the anaerobic chamber to make 100 mL of MS medium. The flask was left in the anaerobic chamber overnight to de-oxygenate.

(iii) MSF medium for *M. formicicum*

MSF medium is simply MS medium (see above) to which 1 mL of 2.5% sodium formate was added in the anaerobic chamber, followed by de-oxygenation.

(iv) MSH medium for *M. maripaludis*

MSH medium is simply MS medium (see above) to which 2.95 g of NaCl, 0.17 g of $MgCl_2$,and 0.05 g of KCl was added, followed by de-oxygenation.

The following day, 10 mL of each growth media were then transferred into anaerobic culture tubes inside the anaerobic chamber, making 10 tubes of each type of medium. The tubes

were then sealed with butyl rubber stoppers, removed from the chamber, crimped with aluminum caps, and autoclaved for sterilization.

1.2.4 Preparation of Stock Cultures of *M. wolfeii, M. barkeri, M. formicicum,* and *M. maripaludis*

A sterile sodium sulfide solution (2.5% wt/vol; 0.15mL per 10 mL of media) was added to the desired number of media tubes about an hour prior to inoculation of any methanogens in order to eliminate any residual molecular oxygen from the tubes. One half milliliter of methanogens from the stock cultures was inoculated into each of their respective media tubes. Following inoculation, the tubes were pressurized with 2 atm of hydrogen gas and incubated at their optimal growth temperatures—55^{o}C for *M. wolfeii*. 37^{o}C for *M. barkeri*, 37^{o}C for *M. formicicum*, and 25^{o}C for *M. maripaludis*.

1.3 Motivation, Objectives, and Goals of this Study:

Methane has been detected in the Martian atmosphere. However, the source/sources of methane in the atmosphere is/are still unknown. Various potential sources of methane on Mars have been suggested such as volcanic, meteoritic, cometary, hydrogeochemical and biogenic. In order to understand the potential sources of methane on Mars, various techniques need to be utilized. Stable carbon isotope fractionation of methane is one of the important tools that can be used to detect probable sources of methane on Mars. As discussed above, methanogens are considered model life forms on Mars. Now the questions that should be answered: what are the carbon isotopic fractionation patterns of methane produced by methanogens if they are growing on different Mars regolith analogs? Are the carbon isotopic fractionation patterns different on

different types of Mars analogs? How do the different phases of growth of methanogenic archaea affect the carbon isotopic fractionation data?

One challenge for the possibility of life on the surface of Mars is the high flux of DNA damaging ultraviolet radiation (UVC). Therefore, the questions that need to be investigated are: What is the sensitivity of methanogens to UVC radiation? Can the presence of any element, compound, or water in the growth-supporting media protect the methanogens from UVC radiation?

As we know the surface conditions of Mars are extremely hostile for life due to several factors such as low pressure, low temperature, and presence of acidic compounds. However, the subsurface of Mars could have higher pressure, higher temperature, and absence of other stress factors for life. Like Earth's extremophiles, any Martian life form would have to contend with a wide range of physiological factors such as non-optimal pH, pressure, and temperature. Hence, methanogens, which are considered as model microorganisms for life on Mars, the questions that arise are: Can *Methanothermobacter wolfeii,* which is a thermophilic methanogenic archaea, endure acidic and alkaline environments? Can these methanogens survive or grow in environments with non-optimal pressures and non-optimal temperatures?

To understand all the aforementioned questions, the following goals were established:

Goal 1: *Identify and characterize the stable carbon isotope fractionation of metabolic methane produced by methanogens growing on different Mars regolith analogs*. (Chapter 2)

In order to achieve this goal, three different strains of methanogenic archaea were grown on four different types of Mars regolith analogs. The headspace gas samples were then analyzed for methane concentration and stable carbon isotope fractionation using a gas chromatograph and

a cavity ringdown spectrometer, respectively. This research can provide a piece of information towards the big enigma of sources of methane on Mars.

Goal 2: To determine the *sensitivity of hydrated and desiccated cultures of halophilic and non-halophilic methanogenic archaea to ultraviolet radiation (UVC).* (Chapter 3)

To achieve this goal, liquid and desiccated cultures of *Methanococcus maripaludis—a* halophilic methanogenic archaea and *Methanobacterium formicicum*—a non-halophilic methanogenic archaea were irradiated with a wide range of UVC (254 nm) flux simulating Martian UVC flux conditions. The survivability of methanogens was then examined by measuring the methane concentration in the headspace gas samples using a gas chromatograph. Methanogens and halophiles have been considered the most primitive microorganisms on early Earth. Therefore, the study of the halophilic methanogenic archaea not only provides the insight into the possibility of life on Mars, but also provides understanding of the evolution of life during early UV irradiated Earth.

Goal 3: To examine the e*ffects of several physical and chemical factors such as pressure, temperature, pH, and hydrogen concentration on the growth and survivability of methanogens.*

To accomplish this, the goal was further divided into three smaller objectives:

Objective 3a: To investigate the *effect of temperatures on the growth of Methanothermobacter wolfeii in the presence and absence of pressurized hydrogen.* (Chapter 4)

Objective 3b: To investigate the e*ffect of temperature and pressure on the growth and survivability of Methanothermobacter wolfeii and stable carbon isotope fractionation.* (Chapter 5)

In objectives 3a, and 3b *Methanothermobacter wolfeii*, a thermophilic methanogenic archaea, was used. In objective 3a, methanogens were grown in the absence and presence of

200KPa of pressurized hydrogen and incubated at four different temperatures—45^0, 55^0, 65^0, and 75^0C. In objective 3b, which combined pressure and temperature experiments, methanogens were exposed to pressures—1, 400, 800, and 1200 atm—and temperatures—45^0, 55^0, and 65^0C. After exposure to these pressures and temperatures, methanogens were incubated at their optimal growth temperature, 55^0C.

In these experiments, the growth and survivability of methanogens were demonstrated by measuring methane concentration in the headspace gas samples using a gas chromatograph. The effect of combined pressure and temperature on the carbon isotope fractionation of methane was also determined using a Cavity Ringdown Spectrometer. The results suggest methanogens could be plausible life forms in the near subsurface or deep subsurface environments of Mars.

Objective 3c: To investigate the *effect of combined pH, pressure, and temperature on the survivability, growth rate, and morphology of a thermophilic methanogenic archaea.* (Chapter 6)

In order to achieve this objective, *Methanothermobacter wolfeii*, a thermophilic methanogenic archaea was exposed to a wide range of pHs—5, 6, 7, 8, and 9—and pressures—1, 400, 800, and 1200 atm at their optimal growth temperature, 55^0C. The survivability and growth rate were examined by measuring the methane concentration in the headspace gas samples using a gas chromatograph. The phase contrast images of the cells were also taken in different conditions in order to understand the effect of non-optimal pHs and pressures on the morphology of methanogens. The importance of this work is to understand the survivability, growth kinetics, and morphological alteration due to exposure to combined pH, pressure, and temperature on methanogens. This research suggests that the search for life on Mars should not be limited to the surface or near subsurface environment, but also the deep subsurface of Mars.

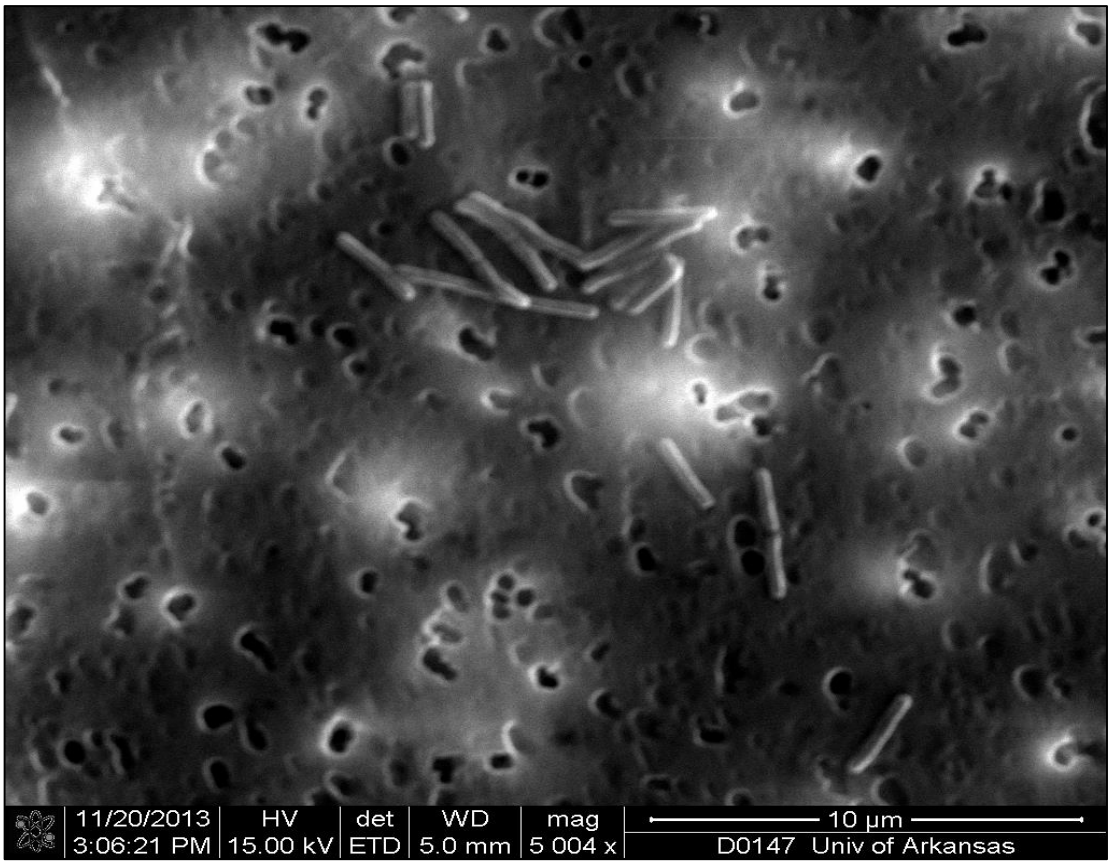

Figure 1.1: A scanning electron micrograph of the *Methanothermobacter wolfeii* (Photo by Navita Sinha)

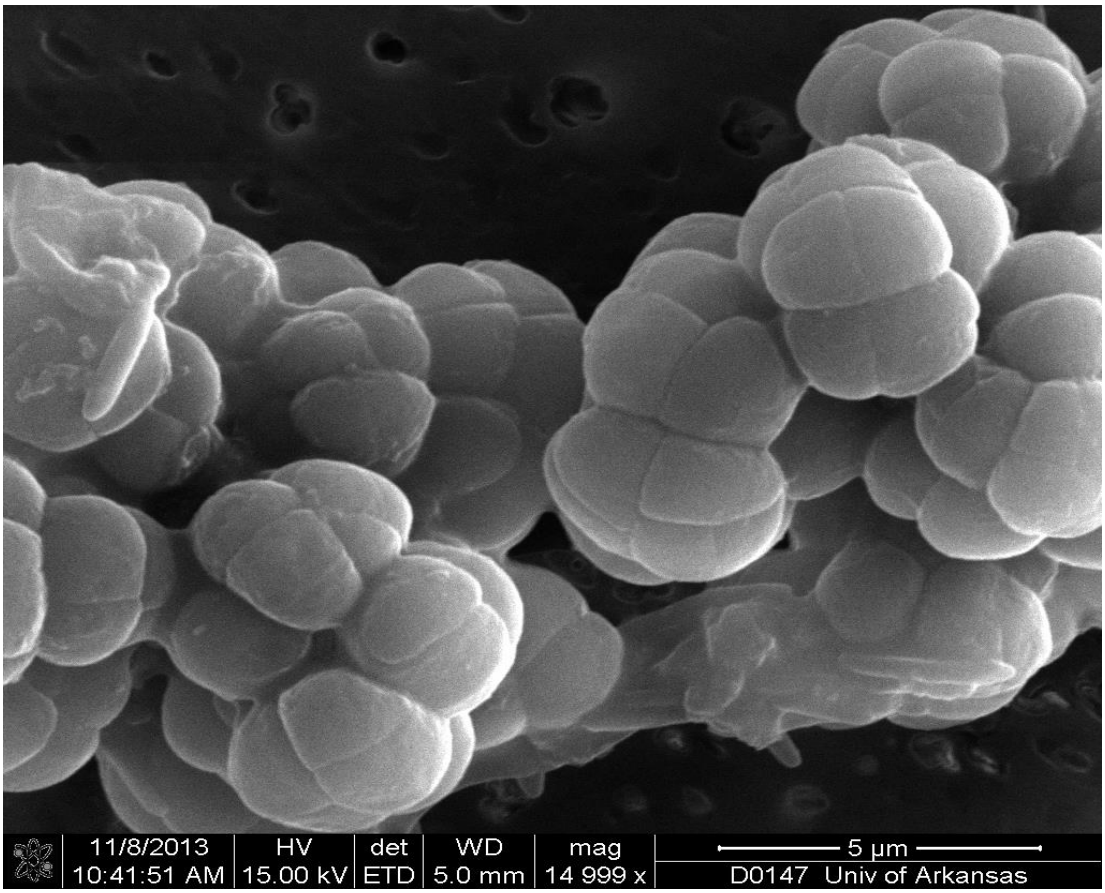

Figure 1.2: A scanning electron micrograph of the *Methanosarcina barkeri* (Photo by Navita Sinha)

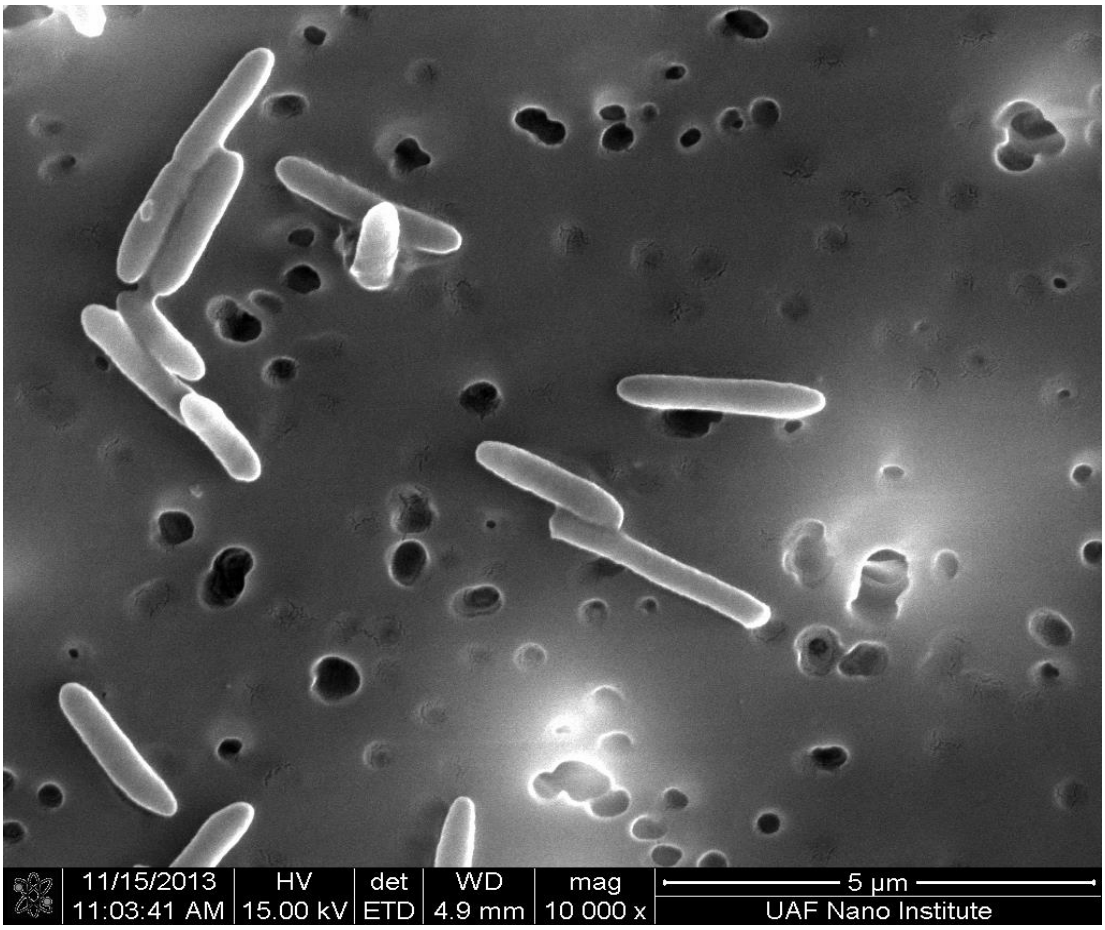

Figure 1.3: A scanning electron micrograph of the *Methanobacterium formicicum* (Photo by Navita Sinha)

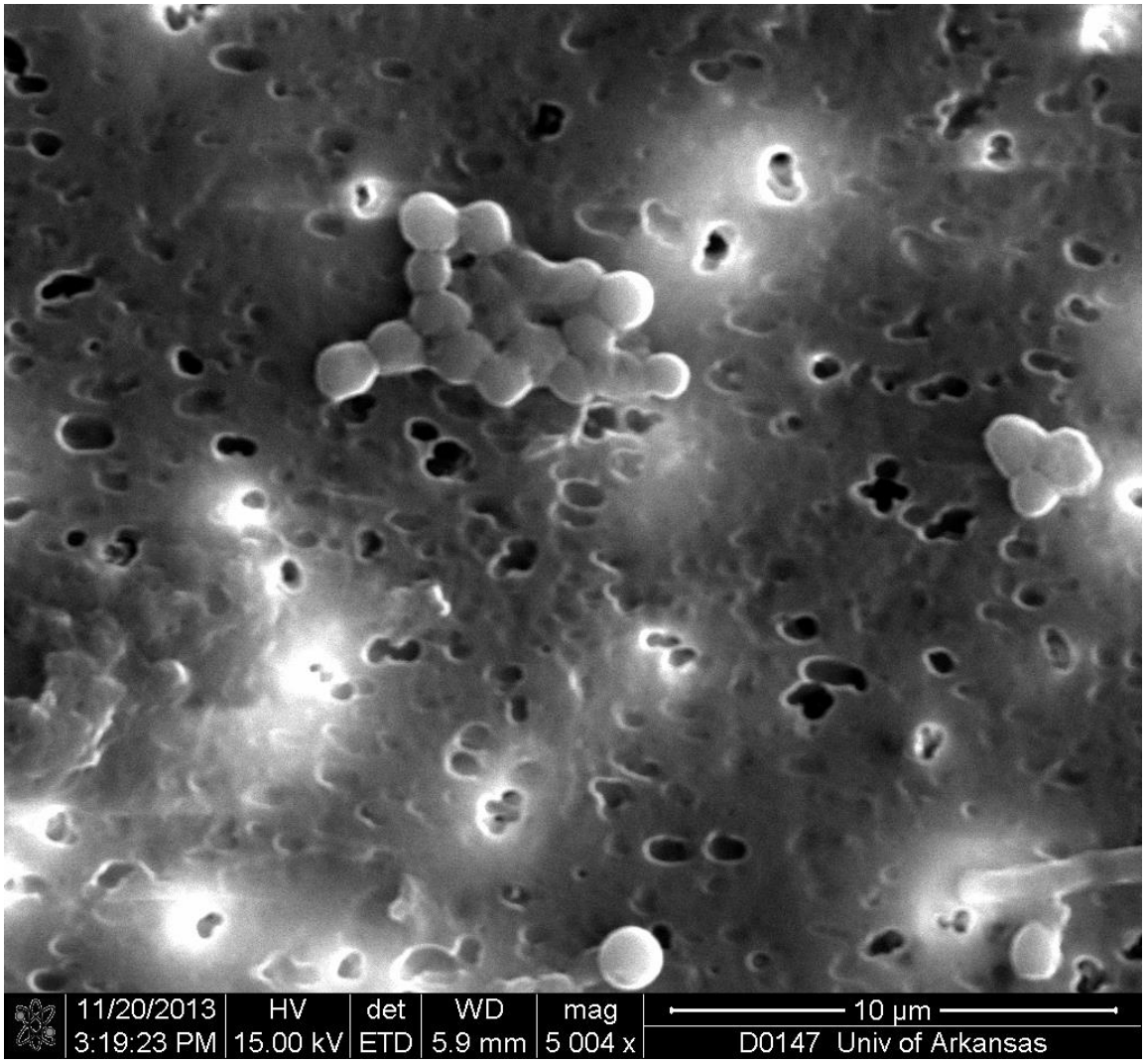

Figure 1.4: A scanning electron micrograph of the *Methanococcus maripaludis* (Photo by Navita Sinha)

Table 1.1: Physiological characteristics of *M. wolfeii*, *M. barkeri*, *M. formicicum*, and *M. maripaludis* (Ferry, 2012)

Property	***M. wolfeii***	***M. barkeri***	***M. formicicum***	***M. maripaludis***
Morphology	Rod	Large and small aggregates	Rod	Cocci
Cell size (microns)	2.4-2.7		2-15	1.0-1.2
Gram stain	Positive	Positive	Positive	Negative
Motility	Non-motile	Non-motile	Non-motile	Motile
pH: optimum	7.0-7.5	5-7	6.6-7.8	7-9
T(ºC): optimum	55-65	35-42	37-40	35-40
Substrates	H_2 and Co_2	H_2, Me, ac	H_2 and formate	H_2 and formate
NaCl(M): optimum range	Not known	<0.2	Not known	0.4
Endospore formation	None	None	None	None
Mol% (G+C)	61(Tm)	39-44 (Bd)	41-42 (Bd)	33 (Bd)
Culture collection	OCM36	OCM38	OCM55	OCM151

Me = Methylated C1 compounds such as methanol, methyl amine, dimethyamine

ac = acetate

Tm = Determined by melting point, Bd = Buoyant density

OCM = Oregon Collection of Methanogens, OR, USA

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Chapter 2

Stable Carbon Isotope Fractionation by Methanogens Growing on different Mars Regolith Analogs

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2.1 Abstract

In order to characterize stable carbon ($^{13}C/^{12}C$) isotope fractionation of metabolically produced methane by methanogens in martian settings, *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* were cultured on four different Mars regolith analogs — JSC Mars-1, Artificial Mars Simulant, montmorillonite, and Mojave Mars Simulant — and also in their growth supporting media. These chemoautotrophic methanogens utilize CO_2 for their carbon source and H_2 for their energy source. When compared to the carbon isotope signature of methane when grown on their respective growth media, *M. wolfeii* and *M. barkeri* demonstrated variability in carbon isotope fractionation values during methanogenesis on the Mars analogs, while *M. formicicum* showed subtle or negligible difference in carbon isotope fractionation values. Interestingly, *M. wolfeii* and *M. barkeri* have shown relatively consistent enriched values of ^{12}C on montmorillonite, a kind of clay found on Mars, compared to other Mars regolith analogs. In general, *M. barkeri* showed large carbon isotope fractionation compared to *M. wolfeii* and *M. formicicum* during methanognesis on various kinds of analogs. Stable carbon isotope fractionation is one of the techniques used to infer different origins, environments, and pathways of methanogensis. The results obtained in this novel research can provide clues to determine ambiguous sources of methane on Mars.

2.2 Introduction

Mars is a cold, arid, and seemingly inhospitable planet. The detection of methane in the martian atmosphere (Formisano et al., 2004; Kranopolsky et al., 2004; Mumma et al., 2009; Webster et al., 2014) and the probability of the existence of liquid water during the early history of Mars (Christensen et al., 2004; Herkenhoff et al., 2004; Klingerhofer et al., 2004; Rieder et al., 2004; Squyres et al., 2006; Ehlmann et al., 2011) and possibly today (McEwen et al., 2011; McEwen et al., 2013) prompts enthusiasm about plausible life forms on Mars. Mumma et al. (2009) have obtained temporal, spatial, and seasonal distribution of methane in the Martian atmosphere. The half-life of methane in a planetary atmosphere is about 300 years (Hunten, 1979), so in order to be detected on Mars, methane would need to be replenished continuously.

Methane on Mars may be ancient or contemporary. If ancient, it may have been stored in clathrate ices and released into the atmosphere from time to time as environmental conditions changed (Chastain and Chevrier, 2007). Various potential sources of methane in the martian atmosphere have been suggested, such as volcanic, meteoritic, cometary, hydrogeochemical, and biogenic sources (Atreya et al., 2007). On Earth, however, about 90 to 95% of atmospheric methane has a biological origin, either from living organisms or decay of organic matter (Atreya et al., 2007). Hence, one explanation for the finding and non-uniform distribution of methane on Mars could be localized microbial sources, either extinct or extant, such as methanogens. Methanogens are anaerobic chemoautotrophs that mostly consume CO_2 as a carbon source and H_2 as an energy source and produce methane as an end product of metabolism.

Methanogens have been considered models for possible martian life-forms even before the discovery of methane in Mars' atmosphere (Boston et al., 1992; Chapelle et al., 2002; Kral et al., 1998; Weiss et al., 2000). Methanogens are anaerobes, and certain strains can tolerate low

pressure, desiccation (Kral et al., 2011), and very cold temperature (Reid et al., 2006), like conditions present on Mars. Additionally, CO_2 is abundant in the Martian atmosphere while molecular hydrogen could be formed by serpentinization on Mars (Oze and Sharma, 2005; Lyons et al., 2005; Schulte et al., 2006). These gases could provide both a carbon and an energy source for methanogens.

To determine if methanogens could be plausible extinct or extant life on Mars, various experimental techniques need to be utilized. Stable carbon isotope fractionation is one of the important techniques that can suggest potential sources of methane (Allen et. al., 2006). Due to difference in mass between isotopes of the same element, partial separation of isotopes occurs during any physical or chemical process. The magnitude of the fractionation depends on the associated isotopic effect of an element in any system. Hence, carbon isotope fractionation values aid in recognition of the surroundings of methanogenesis, such as type and availability of different types of substrates, as these factors have an effect on carbon isotope fractionation of methane (Londry et al., 2008). Life, as we know it, tends to favor the lighter isotope of carbon, carbon-12, because of the lower energy costs during the bond formation in any biochemical reactions. Consequently, bioorganic molecules are rich in carbon-12 but deficient in carbon-13. Due to this, carbon isotopic fractionation may be able to distinguish between biologically and geologically produced methane. Moreover, carbon isotope composition, along with hydrogen isotope composition, may possibly discriminate microbial methane from thermogenic methane, such as methane produced by thermal decay of complex organic matter (Allen et al., 2006; Schoell M., 1988).

The carbon isotope fractionation, $\delta^{13}C$, value allows an understanding of different pathways of methanogenesis. Microbial methane usually has $\delta^{13}C(CH_4)$ lower than -60‰,

whereas thermogenic methane has $\delta^{13}C(CH_4)$ higher than -50‰ (Cicerone and Oremland, 1988). Moreover, $\delta^{13}C(CH_4)$ between -110‰ to -60‰ is the result of autotrophic methanogenesis, while $\delta^{13}C(CH_4)$ ranging between -65‰ to -50‰ is the result of acetotrophic methanogenesis (Whiticar et al., 1986). Hence, the characterization of the carbon isotope fractionation of methane is crucial to identify the potential sources of methane on Mars.

In this paper, we present the carbon isotope fractionation pattern of methane produced by three different strains of methanogens growing on four different Mars regolith analogs. These methanogens have shown survivability on these analogs (see *Materials and Methods*) in previous studies (Kral et al., 2004; Chastain et al., 2010). In this work, methanogens were provided CO_2, in the form of bicarbonate buffer, and molecular hydrogen in the gaseous form. Molecular hydrogen acts as an electron donor and reduces CO_2 to methane. Micronutrients were provided by the regolith analogs.

The carbon isotope fractionations of methane by methanogens on Earth have been studied for a long time in both natural and carefully controlled laboratory environments. The nature of the stable carbon isotope fractionation of methane by methanogens growing on martian regolith simulants has not been studied so far. Therefore, this novel work attempts to further our understanding of the source of methane on Mars.

2.3 Materials and Methods

2.3.1 Methanogenic Cultures and Growth media

Pure cultures of *Methanothermobacter wolfeii* (OCM36), *Methanosarcina barkeri* (OCM38), and *Methanobacterium formicicum* (OCM55) were initially obtained from Oregon

Collection of Methanogens, Portland State University, Portland, OR. Each species was grown in its respective growth supporting medium:

(i) MS medium (Boone et al., 1989) for *M. barkeri,* which contains yeast extract, trypticase peptone, mercaptoethanesulfonic acid, potassium phosphate, ammonium chloride, calcium chloride, resazurin as an oxygen indicator, and many trace minerals*;*

(ii) MSF medium for *M. formicicum,* which contains the same composition as MS medium but also includes sodium formate;

(iii) **MM medium** (Xun et al., 1988) for *M. wolfeii,* which contains the same components as MS medium except yeast extract, trypticase peptone, and mercaptoethanesulfonic acid.

These media were prepared in a Coy Laboratories anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen. Different growth media were then transferred into anaerobic culture bottles inside the anaerobic chamber as described by Boone et al. (1989). These containers were then sealed with butyl rubber stoppers, removed from the chamber, crimped with an aluminum cap, and autoclaved for sterilization.

In order to eliminate any residual molecular oxygen from the vessels containing the media, a sterile sodium sulfide solution (2.5% wt/vol; 1.5 mL per 100 mL of media) was added to each vessel about an hour prior to inoculation of the methanogens (Boone et al., 1989). The vessels were pressurized with 200 kPa of hydrogen gas and incubated at their respective optimal growth temperatures. *M. wolfeii* grows optimally around 55^0C, while the optimal growth temperature for *M. barkeri* and *M. formicicum* is around 37^0C.

2.3.2 Mars Regolith Analog Substrate Preparation

Four different Mars regolith analogs utilized in this experiment were JSC Mars-1 (Allen et al., 1998); Artificial Mars Simulant (AMS) (Fanale et al., 1982), which is a mixture of 45% smectite, 45% basalt, and 10% hematite; montmorillonite (Bentonite, WA: 46E 0438, size < 63 µm); and Mojave Mars Simulant (MMS; Peters et al., 2008), which mainly consists of basalt. Montmorillonite, a clay mineral, is abundant on Mars (Bishop and Murad, 2004). A total of thirty-six 150 mL serum bottles were used for three different strains of methanogens and four different Mars simulants. For statistical sampling, each experiment was done in triplicate. Four bottles were also prepared for negative controls consisting of regolith simulant without methanogens. Each bottle contained 3g of the regolith analog. They were left overnight in the anaerobic chamber to deoxygenate. On the following day, 60 mL of bicarbonate buffer were added to each bottle. Bottles were sealed with butyl rubber stoppers, removed from the chamber, secured with aluminum crimps and autoclaved. For positive controls, three bottles of each medium containing 60 mL of MM, MS, and MSF were also prepared. Another three bottles of a negative control consisting of only sterile buffer were also utilized.

2.3.3 Inoculation of Methanogens in Growth Media and Mars Analogs

Actively growing microbial cells were centrifuged at 6000 rpm (Beckman CP centrifuge) for 10 min and washed two times with reduced sterile bicarbonate buffer in sterile plastic centrifuge tubes. McAllister and Kral (2006) determined that these methanogen strains could tolerate up to 1.5 hours of exposure to atmospheric oxygen during the washing procedure. Washing of cells with buffer ensures that they do not carry over any residual growth media. The washed cells of each species were then suspended in 15 mL of sterile bicarbonate buffer. Each

bottle containing a Mars regolith analog received a 1 mL aliquot of their respective cell suspension (except negative control bottles). All bottles, except buffer-containing bottles, were pressurized with 200 kPa of H_2 and incubated at the organisms' respective optimal growth temperatures.

Headspace gas was analyzed periodically for methane concentration using a Varian CP-4900 Micro-GC. Stable carbon isotopic fractionation of the methane and starting CO_2 was measured by a Cavity Ringdown Spectrometer G2201 (University of Arkansas Stable Isotope Laboratory).

2.3.4 Isotopic Calculations

Methanogens produce CH_4 as the final end product of catabolic metabolism, using H_2 as an electron donor:

$$CO_2 + H_2 \longrightarrow CH_4$$

In order to calculate the carbon isotope fractionation, $\delta^{13}C$, of methane or carbon dioxide in a sample, a known reference material is used. The carbon isotope fractionation, $\delta^{13}C$, is calculated using the following equation:

$$\boldsymbol{\delta}^{13}\mathbf{C}_{Sample} = \left\{ \frac{\left(\frac{^{13}\mathbf{C}}{^{12}\mathbf{C}}\right)_{Sample}}{\left(\frac{^{13}\mathbf{C}}{^{12}\mathbf{C}}\right)_{Reference}} \mathbf{-1} \right\} \mathbf{*1000}$$

The reference isotopic standard for $\delta^{13}C$ is PDB (Pee Dee Belemnite; O'Leary, 1981). The δ is measured in terms of parts per thousand, or "per mil," and is expressed as ‰.

2.4 Results

2.4.1 Methane Concentration during Methanogenesis:

All three methanogens demonstrated growth by producing methane on all four different Martian regolith analogs. *M. wolfeii*, *M. barkeri*, and *M. formicicum* demonstrated substantial headspace methane concentration on JSC Mars-1 and montmorillonite, as well as in their respective media controls over two months of study. However, these methanogens exhibited relatively less methane in the headspace gas samples when they were grown on AMS and MMS (Figs. 2.1A, 2.1C, and 2.1E). The two sets of negative controls did not show methane at all, as expected. *M. barkeri* produced methane at a slower rate than *M. wolfeii* and *M. formicicum* in standard growth media and on martian regolith analogs.

All experimental methanogenic culture bottles used in this study were in closed systems, in which growth of the methanogens and methane production depended on the availability of the substrates such as CO_2, H_2, and micronutrients from analogs.

2.4.2 Isotopic Analysis

Carbon isotope fractionation of methane produced by these methanogens was measured every three days for 30 days. In that time period, there were very small decrease in the carbon isotopic values in some conditions. Therefore, it was decided to measure carbon isotope signature again after an additional month. We did not find any obvious change during this additional 30 days.

M. wolfeii, *M. barkeri*, and *M. formicicum* revealed different magnitudes of carbon isotope fractionation on different substrates. The methane produced by *M. wolfeii* and *M. barkeri* demonstrated relatively depleted ^{13}C when they were cultured on JSC Mars-1, AMS,

montmorillonite, and MMS, compared to their respective growth media controls (Figs. 2.1B and 2.1D). In biogenic methanogenesis, carbon fractionation is due to preferential uptake of lighter isotopes (^{12}C) because of the lower energy costs (O'Leary, 1981). Samples with lower $\delta^{13}C$ values indicate that they are relatively more enriched in the lighter isotope than in the standard. Progressive enrichment of ^{12}C can be seen in Figs. 2.1B and 2.1D, in which the slopes of lines are slowly decreasing.

M. formicicum, when grown in medium and on four different Mars analogs, demonstrated little or no difference in their carbon isotopic fractionation values. $\delta^{13}C(CH_4)$ values ranged between -69.12‰ and -75.6 ‰ on the four different Mars analogs. These values are not very different from the $\delta^{13}C$ value in their growth media, which ranged between -74.44 ‰ and -71.66 ‰ (Fig. 2.1F).

M. wolfeii consistently showed relatively lower carbon isotope fractionation values of around -83‰ on montmorillonite over sixty days of study, while on JSC Mars-1, AMS, and MMS, these values varied between -77.87 ‰ and -73.75‰ (Fig. 2.1B).

Like *M. wolfeii, M. barkeri* also exhibited comparatively lower carbon isotope fractionation of about -90‰ on montmorillonite, but it also showed a lower fractionation pattern on MMS of about -88‰. The $\delta^{13}C(CH_4)$ values on JSC Mars-1 varied from -88.12 ‰ to -82.7‰, and from -89.03 ‰ to -84.66 ‰ on AMS (Fig. 2.1D). *M. barkeri* seems to preferentially use the lighter isotope of carbon, ^{12}C, compared to *M. wolfeii* and *M. formicicum* (Fig. 2.2). *M. barkeri* showed continuously depleted $\delta^{13}C(CH_4)$ values in the media control and on the Mars analogs (Fig. 2.1D).

Comparing the initial (Day 8) and final (Day 63) set of $\delta^{13}C(CH_4)$ values of *M. wolfeii, M. barkeri,* and *M. formicicum, M. barkeri* exhibited more depleted ^{13}C when they were cultured

on the Mars analogs and in the growth supporting media (Table 2.1). As reactions progress, there is a gradual increase in $\delta^{13}C$ values which means organisms initially prefer to consume lighter isotopes of carbon and to leave behind heavier isotopes in the sample.

The carbon isotope data of initial CO_2 from the negative samples were also measured. The average values for $\delta^{13}C(CO_2)$ of negative samples containing Mars regolith analogs and buffer without methanogens were -40.9 ‰, -39.27 ‰, -40.77 ‰, and -40.96 ‰ on JSC Mars-1, AMS, montmorillonite, and MMS respectively. $\delta^{13}C(CO_2)$ for the negative samples containing only buffer was close to these values, -38.36 ‰.

The differences between the initial and final $\delta^{13}C(CH_4)$) values for *M. wolfeii*, *M. barkeri*, and *M. formicicum* in their respective growth supporting media (control) and on four different types of Mars regolith analogs are shown in Table 2.2 and calculated using the following equation:.

$$\Delta\delta^{13}C = \delta^{13}C(CO_2) - \delta^{13}C(CH_4)$$

The slight decrease in the magnitude of final $\Delta\delta^{13}C$ values is found in all situations, except two cases: *M. barkeri* on Mojave Mars Simulant and *M. formicicum* on montmorillonite. The reason for this is not clear.

2.5 Discussion

2.5.1 Methane Concentration during Methanogenesis:

All three methanogenic archaea demonstrated lower concentrations of methane in the presence of all Mars simulants compared to their respective media controls. The pathways of methanogenesis are very complex and involve several enzymes, cofactors, and coenzymes

(Blaut, 1994). During methanogenesis on Mars analogs, methanogens obtain micronutrients from nutrient-limited Mars soil simulants, while in control experiments methanogens are not nutrient-limited in their respective nutrient-enriched growth media. This same phenomenon was seen previously with these same methanogen strains growing with other substrates (e.g. sand, gravel, basalt) supplying the micronutrients (Kozup and Kral, 2009).

2.5.2 Isotopic Analysis

In order to detect possible extant and/or extinct life on Mars, stable carbon isotope studies are imperative (Rothschild and DesMarais, 1989; Schidlowski, 1992; Allen et al., 2006; Atreya et al. 2007). The carbon isotopic measurement techniques on Earth have been used for a long time in order to understand sources of methane, availability of substrates, environments of methanogenesis and cycling of methane.

However, carbon isotope studies alone cannot provide definite evidence for present or past biological activity on Earth (Sherwood Lollar et al., 2008). Hydrogen and carbon isotope data will contribute to a better understanding of various sources and environments of methanogenesis. These measurements will also help to distinguish between thermogenic and biogenic sources of methane.

Fractionation in any process is the result of either equilibrium or the kinetic isotope effect. Equilibrium isotope fractionation involves the exchange of isotopes of an element in an equilibrium reaction. The reaction rates of any particular isotope in forward and reverse reactions are the same. Conversely, in kinetic isotope fractionation, the reaction rates in forward and reverse directions are not identical. Moreover, in the kinetic reactions the lighter isotopes move and react faster and the product becomes enriched in the lighter isotopes (Kendall C. and

Caldwell E. A., 1998). The carbon isotope fractionation in biological processes is an example of kinetic isotope fractionation (Whiticar, 1999).

Several factors are responsible for the extent of fractionation of carbon, such as species of methanogen, growth rate of methanogen, substrate concentration, isotope composition of substrates, availability of substrates, growth stage of the species, environmental factors, isotopic effects of enzymes involved in biosynthetic pathways, and use of different metabolic pathways by methanogens (Conrad et al., 2011; Londry et al., 2003; Londry et al., 2008; Whiticar, 1999; Conrad, 2005; Valentine et al., 2004; Penning et al., 2005). The shuttling of carbon during methanogenesis involves complex biosynthetic networks. The difference in the rate of fixation of carbon in the metabolic pathways of methanogens could be responsible for the difference in the magnitude of carbon isotope fractionation.

Additionally, the rate and extent of carbon isotope fractionation depend on the rate-limiting step of methanogenesis (Kendall and Caldwell, 1998), which could be different in growth media and on different Mars analogs. The methane concentration on Mars soil simulants increased slowly over time when compared to the increase in methane concentration on growth media (Figs. 2.1A, 2.1C, and 2.1E). Kendall and Caldwell (1998) suggest organisms show larger fractionation when growing more slowly. At slower growth, organisms have more time to be more selective in using lighter isotopes. This is another possible factor as to why methanogens demonstrated larger fractionation on Mars analogs compared to their respective growth media.

Overall, the characterization of carbon isotope fractionation content during methanogenesis on different kinds of Mars analogs represents a step forward toward understanding the ambiguous sources of methane on Mars.

NASA's Curiosity rover has the capability to search for organic compounds and to identify the chemical and isotopic composition of the Martian atmosphere by using the tunable laser spectrometer (TLS) of Sample Analysis at Mars (SAM). Curiosity has been roving in the Gale crater of Mars since August 6, 2012. Recently, Webster et al. (2013) have reported in situ measurements of the isotopic ratios of C and O in Martian carbon dioxide.

However, challenges might arise in analyzing martian carbon isotope fractionation (Rothschild and DesMarais, 1989). For example, carbon isotope fractionation of methane on Earth is calculated with respect to standard inorganic carbon such as belemnite of the Pee Dee Belemnite formation in South Carolina (PDB), which allows for more precise measurement. Thus, it would be necessary, and likely challenging, to find an inorganic reference material on Mars. Another challenge is to determine the carbon isotope ratio of the source carbon, which is used in methanogenesis. Despite these potential challenges, these experimental data may provide clues for understanding sources and environments of methanogenesis on Mars, if microbial methane does indeed exist.

2.6 Conclusions

Methanothermobacter wolfeii, *Methanosarcina barkeri*, and *Methanobacterium formicicum* were grown on four different Mars regolith analogs: JSC Mars-1, Artificial Mars Simulant, Mojave Mars Simulant, and montmorillonite. These methanogens were also grown in their growth-supporting media. Carbon dioxide, in the form of bicarbonate buffer, was provided as a carbon source and gaseous hydrogen was provided as the energy source. Stable carbon ($^{13}C/^{12}C$) isotope fractionation values of methane produced by methanogens as a metabolic byproduct were analyzed.

M. wolfeii and *M. barkeri* both showed variable discrepancy in their carbon isotope fractionation values for each of the regolith analogs, when compared with their respective carbon isotope values for their growth media. On clay, such as montmorillonite, *M. wolfeii*, and *M. barkeri* showed relatively depleted ^{13}C compared to other Mars regolith analogs. *M. barkeri* had shown continuously depleted ^{13}C during methanognesis on various analogs, which suggests that these organisms are very selective of using ^{12}C in media, as well as on Mars analogs. In general, *M. barkeri* showed greater carbon isotope fractionation compared to *M. wolfeii* and *M. formicicum* during methanognesis on the various analogs tested. The reason for this difference is unclear. On Earth, the carbon isotope values of methane are often used to distinguish between various sources and pathways of methanogensis. Therefore, this research can provide a piece of information towards the big enigma of sources of methane on Mars.

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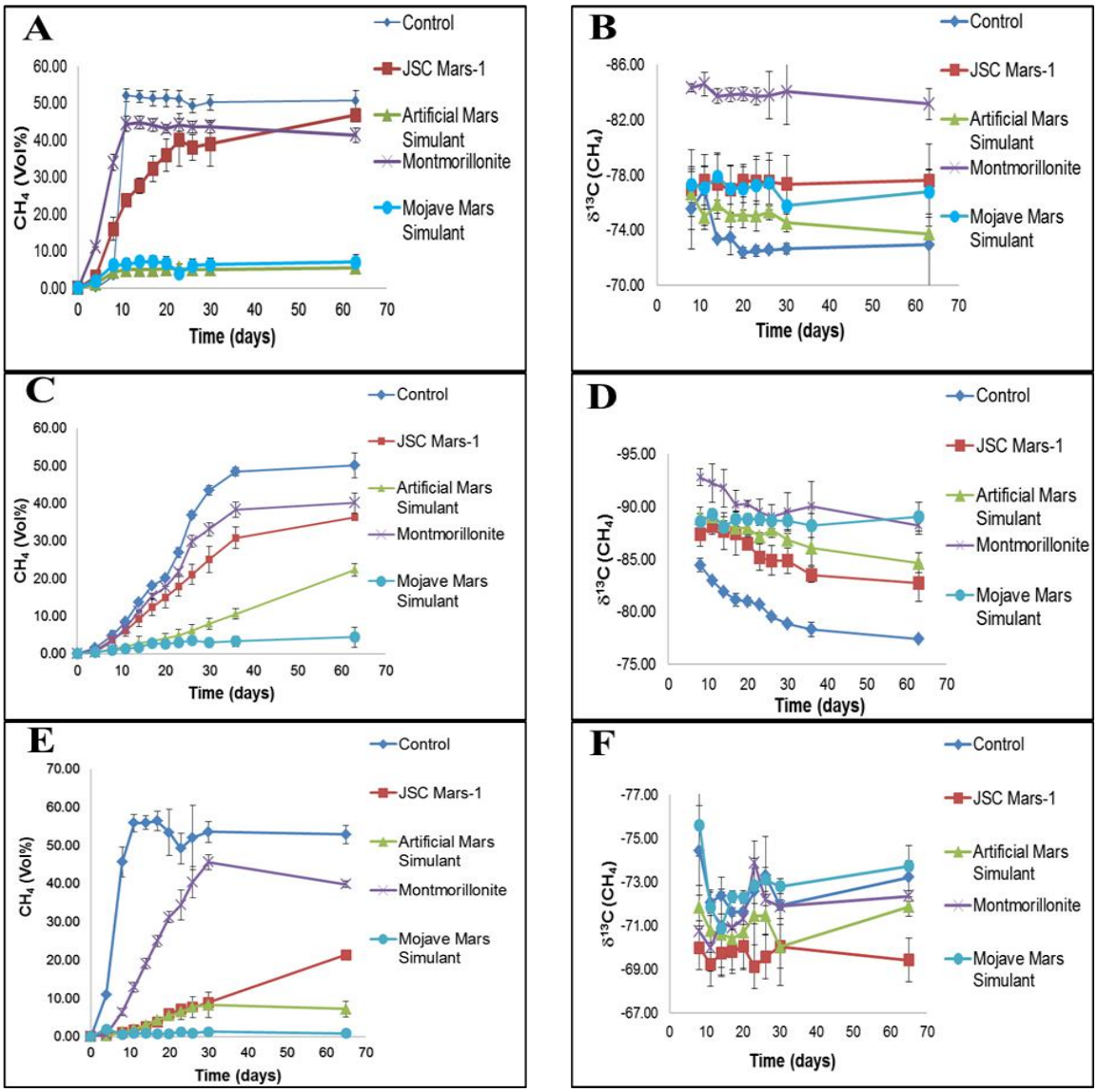

Figure 2.1: Methane concentration as a function of time following methanogenesis on four different Mars regolith analogs and in their respective growth media for three different methanogen species: (A) *Methanothermobacter wolfeii*, (C) *Methanosarcina barkeri*, and (E) *Methanobacterium formicicum* Carbon isotope fractionation values of methane produced during methanogenesis on four different Mars regolith analogs and in their respective growth media for three different methanogen species: (B) *M. wolfeii*, (D) *M. barkeri*, and (F) *M. formicicum*. Each value is the average of three measurements. Error bars represents +/- one standard deviation.

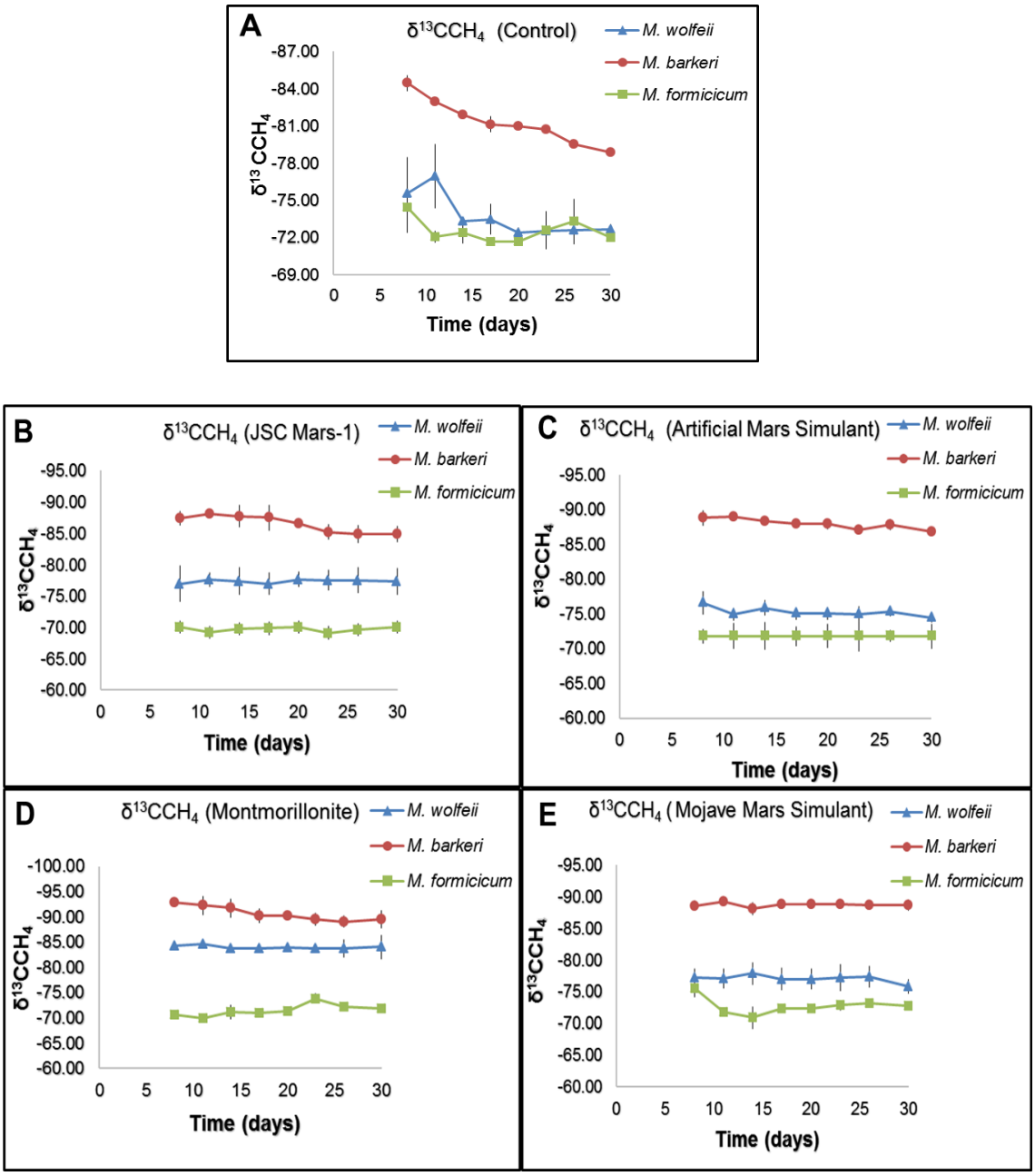

Figure 2.2: Carbon isotope fractionation by *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* as a function of time following methanogenesis in their respective growth media control (A), JSC Mars-1 (B), Artificial Mars Simulant (C), Montmorillonite (D), and Mojave Mars Simulant (E). Each value is the average of three measurements. Error bars represents +/- one standard deviation.

Table 2.1: Initial (Day 8) and final (Day 63) carbon isotope fractionation values of methane produced by *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* in their respective growth supporting media control and four different types of Mars regolith analogs: JSC Mars-1, Artificial Mars Simulant, montmorillonite, and Mojave Mars Simulant. Values shown are in per mil and an average of triplicates.

	M. wolfeii		***M. barkeri***		***M. formicicum***	
	$\delta^{13}C(CH_4)$		**$\delta^{13}C(CH_4)$**		**$\delta^{13}(CCH_4)$**	
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**
Control	-75.54	-72.92	-84.46	-77.42	-74.45	-73.23
JSC Mars-1	-76.92	-77.60	-87.40	-82.79	-70.00	-69.44
Artificial Mars Simulant	-76.63	-73.75	-88.88	-84.67	-71.84	-71.88
Montmorillonite	-84.35	-83.16	-92.81	-88.23	-70.73	-72.37
Mojave Mars Simulant	-77.28	-76.77	-88.61	-89.07	-75.62	-73.74

Table 2.2: Initial and final $\Delta\delta^{13}C$ values, $\delta^{13}C(CO_2) - \delta^{13}C(CH_4)$, of *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* in their respective growth supporting media and four different types of Mars regolith analogs such as JSC Mars-1, Artificial Mars Simulant, montmorillonite, and Mojave Mars Simulant. Values shown are in per mil and an average over triplicates.

	M. wolfeii		***M. barkeri***		***M. formicicum***	
	$\delta^{13}C(CO_2) - \delta^{13}C(CH_4)$		**$\delta^{13}C(CO_2) - \delta^{13}C(CH_4)$**		**$\delta^{13}C(CO_2) - \delta^{13}C(CH_4)$**	
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**
Control	37.18	34.56	46.10	39.06	36.09	34.87
JSC Mars-1	36.02	36.70	46.50	41.89	29.10	28.54
Artificial Mars Simulant	37.36	34.48	49.61	45.40	32.57	31.88
Montmorillonite	43.58	42.39	52.04	47.46	29.96	31.60
Mojave Mars Simulant	36.32	35.81	47.65	48.11	34.66	32.78

2.7 References

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Chapter 3

Effect of UVC Radiation on Hydrated and Desiccated Cultures of Halophilic and Non-Halophilic Methanogenic Archaea: Implications for Life on the Surface of Mars

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Preface: This chapter has been sent for publication and is currently under review.

3.1 Abstract

Methanogens have been considered ideal model life forms on Mars for a long time. In order to survive on the surface of Mars, methanogens would have to endure Martian UVC radiation as well as periods of desiccation. However, the possibility of intermittent briny water on the surface of Mars has been described. Therefore, methanogens on Mars could be in an anhydrobiotic or hydrated state. In this research, we irradiated hydrated and desiccated cultures of halophilic *Methanococcus maripaludis* and non-halophilic *Methanothermobacter formicicum* for time intervals of 0, 1, 2, 4, 8, 12, 16, and 24 hours with UVC (254 nm) radiation in order to examine their survivability. The UVC flux of the lamp was in the range of 7 - 9.9 Wm^{-2} (25.2 – 35.64 $KJm^{-2}\ h^{-1}$). The survivability of the methanogens was determined by measuring methane concentration in the headspace gas sample using a gas chromatograph after re-inoculation of the methanogens into their respective growth-supporting media following exposure to UVC radiation. Hydrated *M. maripaludis* survived for 24 hours of UVC exposure, while in a desiccated condition, they endured for 16 hours. *M. formicicum* also survived UVC radiation for 24 hours in the liquid state, however, in a desiccated condition, the survivability of *M. formicicum* was decreased to 12 hours. Some elements or minerals such as iron, sulfur, sodium chloride, and iron sulfate were present in the growth media and could have served as shielding agents that protected the cells from the damage caused by exposure to the ultraviolet radiation for various time intervals in the hydrated and desiccated conditions. Overall, these results suggest that limited exposure (12 – 24 hours) to UVC radiation on the surface of Mars is not necessarily be a limiting factor for the survivability of *M. maripaludis and M. formicicum.*

3.2 Introduction

3.2.1 Mars UV Radiation

Ultraviolet (UV) flux on a celestial body is one of the significant constraints that affect the evolution of life on that system. The current Martian UV flux is much stronger and harsher (Cockell et al., 2000; Hassler et al., 2014; Patel et al., 2004; Patel et al., 2003; Rontó et al., 2003) than the present Earth UV flux. The reason is that Mars lacks a significant global ozone layer and a magnetic field. The ozone layer in the atmosphere absorbs harmful UV radiation of the Sun and prevents it from reaching the surface, and the magnetic field deflects energetic charged particles such as galactic cosmic rays (GCRs) and solar energetic particles (SEPs) and blocks incidents on the surface. In addition to this, the Martian atmosphere is much thinner than that of the Earth's atmosphere contributing to the higher UV flux on the surface of Mars. The solar constant of Mars is only 43% of that found on Earth mostly in the ultraviolet region of 200-300 nm (Kuhn and Atreya, 1979). Below ~190 nm, the radiation is attenuated by Martian atmospheric CO_2. However, it has been suggested that Earth and Mars shared a similar geological history (Nisbet and Sleep, 2001). Before the rise of oxygen and ozone in the Earth atmosphere, life was probably exposed to higher doses of UV radiation than that of the present day Earth (Kasting, 1993; Sagan, 1973). The DNA damaging UV irradiation on the surface of present day Mars is analogous to that of the Archaean Earth. Hence, UV flux may not be a critical limiting factor for the evolution of life on Mars (Cockell, 1998), but the combination of several environmental factors such as low temperature, lack of liquid water, and the presence of oxidizing compounds cause Mars to be currently inhospitable.

In spite of the hostile surface conditions of present-day Mars, there is a possibility that life could have evolved on early Mars or is still present today. Early Martian life could have left

biosignatures on Mars as well. Furthermore, with the discovery and the study of several types of Earth extremophiles (Rothschild and Mancinelli, 2001) and the occurrence of transient liquid water on Mars (Martin-Torres et al., 2015), it is not prudent to say that life is impossible on the surface of contemporary Mars. Interestingly, the discovery of methane in the Martian atmosphere (Formisano et al., 2004; Mumma et al., 2004; Mumma et al., 2009) further bolstered the perception of the possibility of life on Mars. On Earth, 90-95 % of atmospheric methane has a biological origin (Atreya et al., 2007). One of the sources of Earth's atmospheric methane is methane-producing Archaea called "methanogens." Methanogens have been considered ideal candidates for life on Mars for a long time. Our Exobiology lab research group has been studying the survivability of methanogens in different conditions approaching those on Mars even before the discovery of methane in the Martian atmosphere (Kendrick and Kral, 2006; Kral et al., 2004; Kral et al., 1998).

The chemistry of Mars supports the possibility of life (Sinha and Kral, 2015). The presence of C, H, N, O, P, S, heavy metals, and minerals (Banin and Mancinelli, 1995) provide essential ingredients for the building blocks of life, for example, for the synthesis of proteins and DNA. Additionally, the presence of various forms of salts such as halite, sulfate, and perchlorates found on the surface of Mars (Gooding, 1992; Hecht et al., 2009; Wang et al., 2006) could not only support some forms of life, but could also decrease the freezing point and the evaporation rate of water. For these reasons, salt-loving microorganisms called halophiles are also considered as a model life form on Mars (Litchfield, 1998). Halophiles are classified as extremophiles that can survive and thrive in high salt concentrations and low water activity environments. Even some haloarchaea have been isolated from rock salt samples which are hundreds to millions of years old, (Fendrihan et al., 2007; Mormile et al., 2003) which suggests

that halophiles can survive for a long time under desiccated salty environments. However, the mechanisms responsible for the extreme longevity of archaea in the dormant stage are not known because archaea do not form spores (Grant et al., 1998).

Ultraviolet (UV) radiation wavelength ranges from 4 to 400 nm in an electromagnetic spectrum. The wavelength below 400 nm is harmful, mutagenic or even lethal for most of Earth's life. The short wavelength of UV, such as UV-B (280-315 nm) and UV-C (200-280 nm), causes lesions in DNA molecules of a biological system resulting in temporary or permanent mutations. A covalent bond is formed between two adjacent thymine or cytosine molecules called pyrimidine dimers (Wang, 2012) and the DNA polymerase cannot replicate beyond the site of dimer formation resulting in cell death. On Mars, the UV flux reaching its surface is mostly between ~190-400 nm. Therefore, life on Mars would have to adapt to UV radiation by using DNA repair mechanisms. Different types of DNA repair systems, such as photoreactivation (Wood et al., 1997), excision repair (Wood et al., 1997), and recombination repair (Seitz et al., 1998), have been reported in Archaea. Moreover, the effect of Martian UV radiation on a biological system can be attenuated by the presence of cloud, dust, ice, water, and minerals. Several physical and chemical ultraviolet screening compounds have been reported including halite--solid NaCl, (Cockell and Knowland, 1999; Rothschild, 1990).

A vast body of literature is available about the growth and the survivability of different types of microorganisms under simulated Martian physical and chemical conditions along with ultraviolet radiations (Mancinelli and Klovstad, 2000; Newcombe et al., 2005; Osman et al., 2008). Methanogens and halophiles have been considered Mars model microorganisms and the most primitive microorganisms on Early Earth (Liu and Whitman, 2008; Oren, 2002; Woese et al., 1990). However, the sensitivity of halophilic methanogenic archaea to sole UVC radiation

has not been examined so far. Therefore, the study of the halophilic methanogenic archaea not only provides the insight into the possibility of life on Mars, but also provides understanding for the evolution of life during early UV irradiated Earth. Most methanogenic chemolithoautrophic anaerobic archaea consume CO_2 for their carbon source and H_2 for their energy source and produce CH_4 as their end product of metabolism. The various possible sources of carbon and hydrogen for methanogens on Mars have been discussed previously (Sinha and Kral, 2015).

In this research, we irradiated hydrated and the desiccated cultures of halophilic and non-halophilic methanogenic archaea with a wide range of UVC (254 nm) flux, simulating the Martian UVC flux conditions, to examine their survivability.

3.3 Materials and Methods

3.3.1 Preparation of Growth Media

Pure cultures of *Methanobacterium formicicum* (OCM55) and *Methanococcus maripaludis* (OCM 151) were obtained from the Oregon Collection of Methanogens, Portland State University, Portland. Each species was grown in its respective growth-supporting medium:

(i) MSF medium (Boone et al., 1989) for *M. formicicum* which contains yeast extract, trypticase peptone, mercaptoethanesulfonic acid, potassium phosphate, ammonium chloride, calcium chloride, sodium formate, resazurin as an oxygen indicator, and many trace minerals;

(ii) MSH medium (Ni and Boone, 1991) for *M. maripaludis*, which contains the same composition as MSF medium without sodium formate. It also contains sodium chloride, potassium chloride, and magnesium chloride.

One hundred milliliters of each medium were prepared in a bicarbonate buffer in a flask inside a Coy Laboratories anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen. MSF and MSH

medium were then transferred into anaerobic culture tubes (10 mL in each tube) inside the chamber. These tubes were then sealed with butyl rubber stoppers, removed from the chamber, crimped with an aluminum cap, and autoclaved for sterilization.

3.3.2 Preparation of Stock Cultures of *Methanobacterium formicicum* and *Methanococcus maripaludis*:

Stock cultures of methanogens were maintained by transferring into their respective fresh growth medium every fifteen days. Actively growing methanogenic cells (approximately 0.5 optical density at 600 nm) were utilized for UV irradiation either in solution or desiccation experiments. A sterile sodium sulfide solution (2.5% wt/vol; 0.125 mL per 10 mL of media) was added to each of the MSF and MSH media tubes about an hour prior to inoculation with the methanogens (Boone et al., 1989) in order to eliminate any residual molecular oxygen. One half of a milliliter from the *M. formicicum* or *M. maripaludis* stock was inoculated into each type of medium. After inoculating methanogens into their respective media, the tubes were pressurized with 200 kPa of hydrogen gas and incubated at their optimal growth temperature, 37^0C. Methane concentration in the headspace gas of each sample was measured periodically using a gas chromatograph (Shimadzu 2014, USA).

3.3.3 Preparation of Liquid Methanogenic Cells in Cuvettes for UV Irradiation:

A sterile syringe was used to transfer 1 mL of an actively growing cell suspension (approximately 0.5 OD at 600nm) to a sterile disposable plastic vial inside the anaerobic chamber. From the vial, 200 uL of aliquot were transferred into a plastic cuvette.

3.3.4 Preparation of Desiccated Methanogenic Cells in Cuvettes for UV Irradiation:

From the same plastic vial in the previous section, 200 uL of aliquot were transferred into plastic cuvettes. These cuvettes were placed into a Nalgene Desiccator (150 mm, VWR Scientific Products, Dallas, TX) containing Drierite (anhydrous calcium sulfate, W. A. Hammond Drierite Company, Ltd., Xenia, OH) for about 72 hours. All work was done inside the anaerobic chamber.

3.3.5 Exposure of Hydrated and Desiccated Methanogenic cells to UV Irradiation in an Anaerobic Condition:

A small UVC lamp (UVG-11|compact UV lamp|4 watt|254 nm| P/N 95-0016-14|0.16 Amps|115V~60Hz) was used to irradiate methanogenic cells inside the anaerobic chamber. The dimension of the compact lamp was 7.8L x 2.8W x 2.1D in. (198 x 71 x 53mm). The lamp was placed 3 inches above the cuvette containing a sample. The intensity of UVC flux, measured using a UVC light meter (UVC Light Meter 850010, SPER SCIENTIFIC), was in the range of 7-9.9 Wm^{-2} (25.2 – 35.64 $KJm^{-2}\,h^{-1}$). The cuvette was placed at the same spot where flux was measured in order to calculate accurate flux experienced by the methanogens. The cuvette containing either liquid or desiccated cells was placed below the UVC lamp one after another for various times ranging from 1 hour to 24 hours. All experiments were performed at ambient temperature and pressure. After exposure for the desired time intervals, 1 mL of the respective sterile growth media was added to each cuvette followed by thorough mixing. These 1 mL aliquots were transferred into their respective growth media (duplicate samples for each time of UVC exposure) with the use of a sterile syringe. Inoculated methanogenic media tubes were removed from the anaerobic chamber, pressurized with 200KPa of H_2, and incubated at their

optimum growth temperature, 37^0C. One milliliter of a headspace gas sample was removed periodically to measure methane concentration using a gas chromatograph (Shimadzu Gas Chromatograph, 2014).

3.4 Results

The halophilic methanogen, *M. maripaludis,* while hydrated survived for about 24 hours of UVC exposure, while as desiccated cells, endured for 16 hours (Table 3.1). The non halophilic methanogenic archaea, *M formicicum,* also survived UVC radiation for 24 hours in the hydrated state; however, while desiccated, the survivability of *M. formicicum* was decreased to 12 hours (Table 3.2). The survivability of the methanogens was determined by measuring methane concentration in a headspace gas sample using a gas chromatograph after re-inoculation of methanogens into their respective growth supporting media following exposure to UVC radiation. The samples were in duplicates. The entire set of experiments was replicated twice. All the experiments were performed in the anaerobic chamber for three reasons: (i) Methanogens are anaerobes. (ii) To mimic the Martian surface environment in terms of oxygen. (iii) Oxygen can interfere the effect of UVC radiation on cells. The experimental set up is shown in Figure 3.1. To normalize the experimental protocol in each set of experiments, the same OD and same volume of exponential phase cells of each species in liquid culture were utilized. In the desiccation experiments, we used the same volume of the liquid cultures to prepare desiccated cells.

3.5 Discussion

We studied the effect of simulated Martian UVC radiation on the survivability of hydrated and desiccated cultures of two methanogens. *M. formicicum,* a non-halophile, and *M. maripaludis,* a halophile. Mars is an extremely dry planet and any putative microorganism on the Martian surface could have to withstand a period of desiccation. However, the possibility of intermittent briny water on the surface of Mars has also been described (McEwen et al., 2014). Therefore, life on Mars could be in an anhydrobiotic or a hydrated state. Anhydrobiosis is a condition in which an organism enters into a completely desiccated state with reduced or no metabolic activity. We have reported earlier that *M. maripaludis* survived desiccation for 60 days while *M. formicicum* survived for 120 days at Martian surface or near surface pressure conditions, 6 mbar (Kral et al., 2011).

In order to determine the survival capability of the liquid and the desiccated methanogenic cells under simulated Martian UVC radiation, we exposed cells to the radiation for various time intervals from 1 to 24 hours. Methane concentrations from headspace gas samples following UVC exposure were measured using gas chromatography. Growth of methanogens is typically measured by increases in methane concentration with time (Sowers and Robb, 1995). Both methanogens, *M. formicicum* and *M. maripaludis,* in liquid culture endured UVC radiation for 24 hours (Table 3.3). However, when desiccated, *M. formicicum* and *M. maripaludis* tolerated ultraviolet radiation for 12 and 16 hours, respectively (Table 3.4).

One possible reason for the survivability of methanogens for 24 hours of UVC exposure in the liquid culture could be the presence of suspended solids or elements in the growth media that prevented the damaging effect of the radiation on the cells. Some elements or minerals such as iron, sulfur, solid NaCl, jarosite (iron sulfate) and gypsum (calcium sulfate) act as shielding

agents and protect cells from the damage caused by the ultraviolet radiation (Cockell et al., 2003; Cockell, 1998; Gómez Gómez et al., 2004; Martinez-Frias et al., 2006). Iron sulfate and NaCl were present in the growth media. The salt concentration in the growth media of *M. maripaludis* was only 4%. The presence of salt (approximately 4%) in the MSH medium may have imparted protection to *M. maripaludis* in both liquid medium and the desiccated state. The minerals such as salt, iron compounds, and jarosite are common on the surface on Mars and could serve as protective materials for any putative life form there as well. Also, the presence of any overlying material or regolith could attenuate the effect of radiation on organisms and could increase the potential habitability of life on Mars. Furthermore, the microbes in the upper layer of a microbial mat can also protect microbes beneath them (Martinez-Frias et al., 2006).

We found that the halophili*c* archaea *M. maripaludis* are more tolerant to ultraviolet radiation than non-halophilic *M. formicicum* (Table 3.4).

The actual reasons for the relative tolerance of methanogens to the UVC radiation are not known. One possible explanation could be the difference in their genomic structure and utilization of DNA repair enzymes. Since enzymes require an aqueous environment for activity, one would expect DNA repair to occur only in the hydrated cells.

In this work, we have not attempted to simulate actual Martian surface conditions such as low pressure, low temperature, atmospheric gas composition or the presence of minerals along with the UVC radiation. We considered only one of the stress conditions, UVC radiation. The results presented here illustrate that halophilic and non-halophilic methanogens, in their hydrated state tolerated simulated Martian UVC radiation longer time than in their desiccated state. Future work will examine possible adaptation to UVC radiation by methanogens.

3.6 Conclusions

The research reported here illustrates that hydrated and desiccated cultures of *M. maripaludis* and *M. formicicum* could tolerate simulated Martian UVC flux for limited lengths of time. We irradiated the liquid and desiccated culture of halophilic *M. maripaludis* and non-halophilic *M formicicum* to UVC (254 nm) flux for several time intervals. In the hydrated state, both strains of methanogens survived for 24 hours of UVC exposure while in the desiccated state, *M. maripaludis* endured for about 16 hours and *M. formicicum* survived for 12 hours of radiation. The reasons for the survivability of these methanogens over various time intervals in the hydrated and desiccated conditions are not known. However, some elements or minerals such as iron, sulfur, sodium chloride, and/or iron sulfate in the growth media and/or the activity of DNA repair enzymes could have protected cells from the damage normally caused by ultraviolet radiation. Therefore, limited exposure (12 – 24 hours) to UVC radiation on the surface of Mars is not necessarily a limiting factor for the survivability of *M. maripaludis and M. formicicum.*

Table 3.1: Survival of hydrated and desiccated *Methanococcus maripaludis* following exposure to various time intervals of UVC radiation.

Methanococcus maripaludis culture in different states	**Time (Hour)**						
	1	**2**	**4**	**8**	**12**	**16**	**24**
Liquid	+	+	+	+	+	+	+
Desiccated	+	+	+	+	+	+	-

+ Methane formation. – No methane formation

Table 3.2: Survival of hydrated and desiccated *Methanobacterium formicicum* following exposure to various time intervals of UVC radiation.

Methanobacterium formicicum in different states	**Time (Hour)**						
	1	**2**	**4**	**8**	**12**	**16**	**24**
Liquid	+	+	+	+	+	+	+
Desiccated	+	+	+	+	+	-	-

+ Methane formation. – No methane formation

Table 3.3: A comparison of the hydrated cultures of *M. maripaludis* and *M. formicicum* following exposure to various time intervals of UVC radiation.

Organism	Time (Hour)						
	1	**2**	**4**	**8**	**12**	**16**	**24**
Methanococcus maripaludis	+	+	+	+	+	+	+
Methanobacterium formicicum	+	+	+	+	+	+	+

+ Methane formation. – No methane formation

Table 3.4: A comparison of the desiccated *M. maripaludis* and *M. formicicum* following exposure to various time intervals of UVC radiation.

Organism	Time (Hour)						
	1	**2**	**4**	**8**	**12**	**16**	**24**
Methanococcus maripaludis	+	+	+	+	+	+	-
Methanobacterium formicicum	+	+	+	+	+	-	-

+ Methane formation. – No methane formation

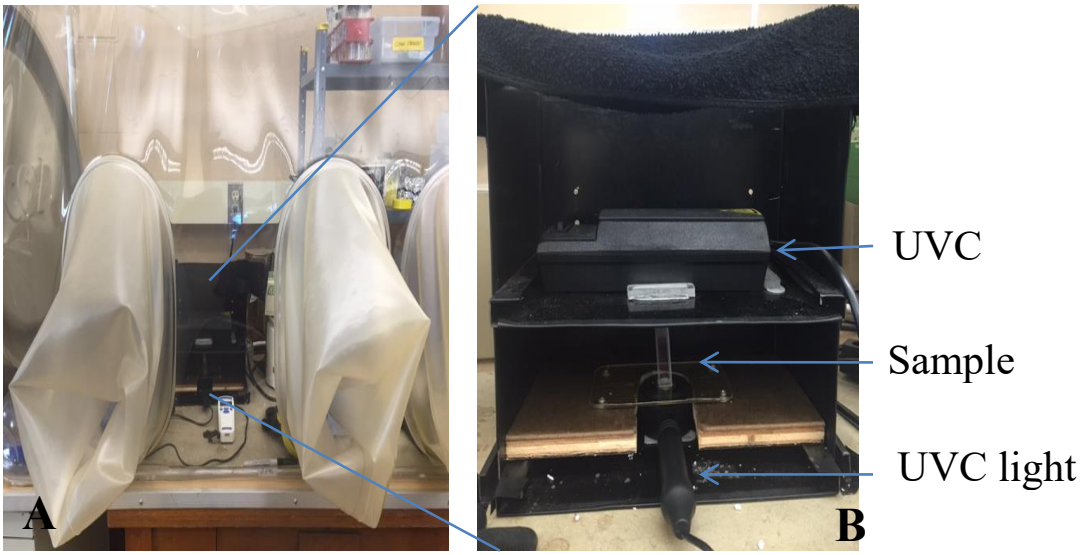

Figure 3.1 (A) Experimental set up inside a Coy anaerobic chamber and (B) Close-up view of the experimental set up.

3.7 References

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Chapter 4

Effect of Temperature on the Growth of *Methanothermobacter wolfeii* in the Presence and Absence of Pressurized hydrogen

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4.1 Abstract

Methanogens, which consume CO_2 for their carbon source and H_2 for their energy source and produce methane, have been considered ideal life forms on Mars. The Martian atmosphere is rich in CO_2. This atmospheric CO_2 could diffuse through the Mars regolith and would be available for methanogenesis. However, the availability of another substrate, H_2, is questionable. It has been speculated that H_2 could be formed through serpentinization reactions in the subsurface of Mars, analogous to serpentinization in Earth's subsurface. However, the source, availability and concentration of H_2 in the subsurface of Mars are still unknown. In order to understand the effects of concentration of H_2 on hydrogenotrophic methanogenic anaerobic archaea, *Methanothermobacter wolfeii* ($T_{optimum}$= 55^{o}C), we have examined methanogenesis in the presence and absence of pressurized H_2 gas. In the customary protocol, CO_2 is made available to methanogens in the form of bicarbonate buffer and H_2 is provided in the pressurized gaseous form. We have also studied the effect of a broad range of temperatures (45^{o}C, 55^{o}C, 65^{o}C, and 75^{o}C) on methanogenic metabolism of *M. wolfeii* with and without pressurized H_2 gas conditions. Interestingly, *M. wolfeii* demonstrated methane production in all different conditions tested. The highest methane concentration peak obtained at 55^{o}C in pressurized hydrogen conditions, while in the absence of pressurized hydrogen, the highest methane concentration was found at 65^{o}C.

4.2 Introduction

The surface conditions of Mars have been considered harsh for any known life forms. One of the known stress factors for life on the surface of Mars is the cold temperature. The subsurface of Mars could provide warm temperature and be suitable for a biosphere. The availability of substrates for life in the subsurface of Mars is another constraint. The growth and survivability of a microorganism depend on ambient physical and chemical conditions that include environmental temperature and substrate availability. Methanogens have been discussed for a long time as model life form on Mars.

Methanogenic archaea obtain energy anaerobically through methanogenesis and produce methane as a byproduct. The several substrates utilized by different genera of methanogens for their metabolic activities are hydrogen plus carbon dioxide, acetate, acetic acid, methanol, methylamine, and dimethylsulfide. The most common substrates for methanogenesis are hydrogen plus carbon dioxide (used in hydrogenotrophic methanogenesis; Simpson and Whitman, 1993; Thauer et al., 1993) and acetate (used in acetoclastic methanogenesis). Some methanogens, such as *Methanosarcina barkeri*, can grow on more than one type of substrate (Hutten et al., 1980). However, it has been reported that types of methanogenesis in sediments also depend on the temperature. Methanogenesis through acetate fermentation is the predominant pathway during summertime while in wintertime, CO_2 reduction is the predominant pathway for methanogenesis (Martens et al., 1986).

Methanogens can grow in a wide range of temperatures and are classified as extremophiles. For example, *Methanopyrus kandleri*, a hyperthermophilic methanogen, can grow at temperatures as high as 110^{o}C (Kurr et al., 1991) whereas psychrophilic methanogens such as *Methanogenium frididum*, can grow at temperatures as low as -10^{o}C (Cavicchioli, 2002;

Cavicchioli et al., 2000). These microorganisms have environmental adaptation mechanisms that help them to carry out all essential cell functions under the extreme conditions. The molecular chaperon proteins such as heat shock and cold shock proteins are often responsible for the stress response in these types of extremophiles.

The model life forms on Mars are hydrogenotrophic methanogens, which require CO_2 for the carbon source and H_2 for the energy source and produce methane (Kral et al., 2011; Kral et al., 2004; Kral et al., 2015; McAllister and Kral, 2006; Sinha and Kral, 2015). The Martian atmosphere is rich in CO_2, which could permeate through the regolith and be available for methanogens. However, the source of hydrogen on Mars is dubious (Weiss et al., 2000). It has been speculated that hydrogen could be available to methanogens through geochemical serpentinization reactions (Lyons et al., 2005; Oze and Sharma, 2005; Schulte et al., 2006). Nevertheless, the actual origin, existence and concentration of hydrogen in the subsurface of Mars are still unknown.

We examined the growth and survivability of the hydrogenotrophic methanogenic archaea, *Methanothermobacter wolfeii* ($T_{optimum}$=55^{o}C), in the presence and absence of pressurized hydrogen gas in a broad range of temperatures (45^{o}C, 55^{o}C, 65^{o}C, and 75^{o}C).

4.3 Materials and Methods

4.3.1 Preparation of Stock Culture in MM medium

M. wolfeii (OCM36), used in this experiment, was initially obtained from the Oregon Collection of Methanogens, Portland State University, Portland, OR. *M. wolfeii* was grown in MM medium. MM medium was prepared in a bicarbonate buffer and consists of potassium phosphate, ammonium chloride, calcium chloride, resazurin as an oxygen indicator, and many

trace minerals (Xun et al., 1988). The MM medium was prepared in a Coy Laboratories anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen. Inside the anaerobic chamber, the growth media were then transferred into the anaerobic culture tubes. The tubes were then sealed with butyl rubber stoppers, removed from the chamber, crimped with aluminum caps, and autoclaved for sterilization.

In a routine protocol, a sterile sodium sulfide solution was added (2.5% wt/vol; 0.15 mL per 10 mL of media) to the required number of anaerobic MM media tube about an hour prior to inoculation of the methanogens. This was done to eliminate any residual molecular oxygen from the media tubes (Boone et al., 1989). The inoculated media tubes were then pressurized with 200 KPa of hydrogen gas and incubated at the optimal growth temperature of *M. wolfeii*, 55ºC. The stock cultures of *M. wolfeii* were maintained by transferring into fresh MM medium every fifteen days.

4.3.2 Methanogenic Growth Experiment in the Presence of Pressurized Hydrogen at Different Temperatures

One milliliter of fresh stock culture of *M. wolfeii* was inoculated into each of eight anaerobic tubes containing 10 mL of sterile deoxygenated MM media. The tubes were then pressurized with 200 KPa of hydrogen gas and were kept at four different temperatures—45ºC, 55ºC, 65ºC, and 75ºC. The samples were in duplicates. Methane concentration of headspace gas of each sample was measured regularly using a gas chromatograph.

4.3.3 Methanogenic Growth Experiment in the Absence of Pressurized Hydrogen at Different Temperatures

One milliliter of fresh culture of *M. wolfeii* was inoculated into each of eight anaerobic tubes containing 10 mL of sterile deoxygenated MM media. The tubes were kept at four different temperatures—45°C, 55°C, 65°C, and 75°C. Methane concentration of headspace gas of each sample was measured periodically using a gas chromatograph. In these sets, we did not pressurize the tubes with hydrogen gas. As discussed previously, the MM medium was prepared in the anaerobic chamber, which was filled with 90% carbon dioxide and 10% hydrogen. Only dissolved hydrogen in the media was available to methanogens for their metabolism.

4.4 Results

M. wolfeii demonstrated methanogenesis through methane production in the presence and absence of pressurized hydrogen at all temperatures tested.

In Figure 4.1, we show methane concentration as a function of time for the temperatures—45°C, 55°C, 65°C, and 75°C in the presence of pressurized hydrogen. Methanogens produced methane continuously until they reached the stationary phase. The lag phase in the case of 45°C was about 100 hours. At 55°C, which is the optimum growth temperature of *M. wolfeii*, the lag phase was about 24 hours. However, at higher temperatures such as 65°C and 75°C, the lag phases were about 14 hours. This suggests that *M. wolfeii* at 65°C and 75°C started metabolizing sooner and at a faster rate than they metabolized at 45°C and 55°C. Nevertheless, the highest methane concentration peak was found at 64 hours at 55°C.

In Figure 4.2, we show methane concentration as a function of time for the temperatures—45^{o}C, 55^{o}C, 65^{o}C, and 75^{o}C in the absence of pressurized hydrogen. The different colored and patterned lines in the graph demonstrate that *M. wolfeii* grew at least for a few days even in the absence of pressurized hydrogen. The maximum methane concentration point was found at 65^{o}C where methane concentration was about 4%.

4.5 Discussion

M. wolfeii grows optimally in the presence of pressurized (200 KPa) H_2 gas at 55^{o}C. The highest growth rate and methane concentration peak was found at 55^{o}C in the presence of pressurized H_2, as expected (Figure 4.1). However, methanogenesis by *M. wolfeii*, in the absence of pressurized hydrogen and at non-optimal growth temperatures such as 45^{o}C, 65^{o}C, and 75^{o}C, was surprising (Figure 4.2).

In the samples without pressurized hydrogen gas, the only hydrogen available for *M. wolfeii* for their metabolism was dissolved H_2. The MM growth media was prepared in the anaerobic chamber, which was filled with 90% carbon dioxide and 10% hydrogen. The concentration of dissolved hydrogen in MM media was not quantifiable. To measure the concentration of each gas in the headspace gaseous samples, we used a gas chromatograph. In the unpressurized hydrogen gas samples, the concentration of hydrogen was below the detection limit of a gas chromatograph. Therefore, it was not clear how much dissolved hydrogen was present in the MM media, which assisted methanogenic growth for a few days. The continuous increase in methane concentration until it reached stationary phase demonstrated ongoing methanogenesis. For higher temperatures—75^{o}C and 65^{o}C, the highest methane concentration

regime was obtained in 48 hours, while for 55ºC and 45ºC, the maximum methane peak arrived in 64 hours and 196 hours respectively. The highest methane concentration and growth rate of *M. wolfeii* were found at 65ºC in the absence of pressurized H_2 gas. The data presented here suggests that temperature plays an important role in carrying out metabolic functions in addition to the substrate, such as hydrogen.

Temperature can affect enzymatic activity, membrane fluidity, and the transport of nutrients into a cell. An enzyme has a minimum, optimum, and maximum temperature for its activity. The enzyme denatures above the maximum and below the minimum temperatures, and the metabolic activity will either stop or slow down. (Zinder et al., 1984) have found that the acetoclastic methanogens, *Methanosarcina sp.,* demonstrated methane production at the optimum growth temperature between 55ºC to 60ºC, but methanogenesis by *Methanosarcina sp* was completely inhibited at 65ºC. In this work, we have found that at temperatures 65ºC and 55ºC, the methane concentrations were about 4% and 3.5% respectively even in the absence of the substrate hydrogen (there was some dissolved H_2). However, at the highest and lowest temperatures 75ºC and 45ºC, the methane concentration was only about 3%.

Methanogenesis also depends on the availability and concentration of hydrogen. Winfrey et al. have found in their in situ studies that the concentration of methane produced by methanogens depends on the concentration of hydrogen available to microorganisms (Winfrey et al., 1977). They found that the addition of hydrogen stimulates methanogenesis and established that hydrogen is an important rate-limiting factor. Hungate found that methanogenesis in rumen depends on the dissolved hydrogen content (Hungate, 2013).

Analogous to Earth's hydrothermal environments, methanogenesis could be enduring near Mars' hydrothermal systems. Several pieces of evidence such as surface features and

isotopic data of Martian meteorites point to hydrothermal activity on Mars (Brakenridge et al., 1985; Romanek et al., 1994; Shock, 1996; Watson et al., 1994). The availability of one of the substrates for methanogens, CO_2, is not a big issue on Mars. However, the source and concentration of another substrate, hydrogen, is not documented yet. It has been hypothesized that hydrogen could be formed by fluid rock reactions in the subsurface of Mars. It is possible that methanogens could have converted hydrogen into methane analogous to Early Earth (Kral et al., 1998). In this study, we made an effort to understand the methanogenesis by *M. wolfeii* in a very low or negligible concentration of hydrogen and conventional hydrogen concentration conditions in a wide range of temperatures. We have found that *M. wolfeii* exhibited metabolic activities by producing methane for a few days at all temperatures tested even in the absence of pressurized hydrogen.

4.6 Conclusions

Hydrogenotrophic methanogens, which use CO_2 and H_2 and produce methane, have been considered the most appropriate model life forms on Mars. CO_2 is abundant in the Martian atmosphere, which could diffuse through the regolith and be available for methanogenic metabolism. Nevertheless, the availability of hydrogen is uncertain. Analogous to Earth's subsurface environments, hydrogen could be formed through serpentinization reactions in the subsurface of Mars. In this study, we have investigated the effects of concentration of H_2 on the hydrogenotrophic methanogenic anaerobic archaea, *Methanothermobacter wolfeii* ($T_{optimum}$= 55^{o}C), in a wide range of temperatures—45^{o}C, 55^{o}C, 65^{o}C, and 75^{o}C. *M. wolfeii* demonstrated metabolism through methane production in all optimal and non-optimal conditions tested. In

pressurized hydrogen conditions, the methane concentration peak was highest at 55°C, while in the absence of pressurized hydrogen, the highest methane concentration peak was at 65°C.

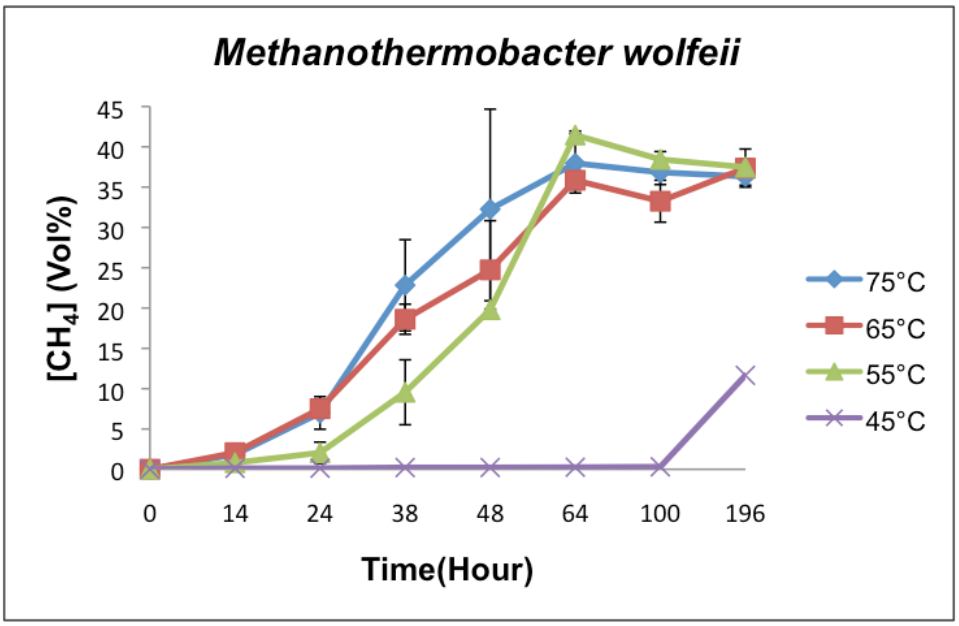

Figure 4.1. Methane concentration as a function of time at temperatures of 45°C, 55°C, 65°C, and 75°C in the presence of pressurized hydrogen gas (2 kPa).

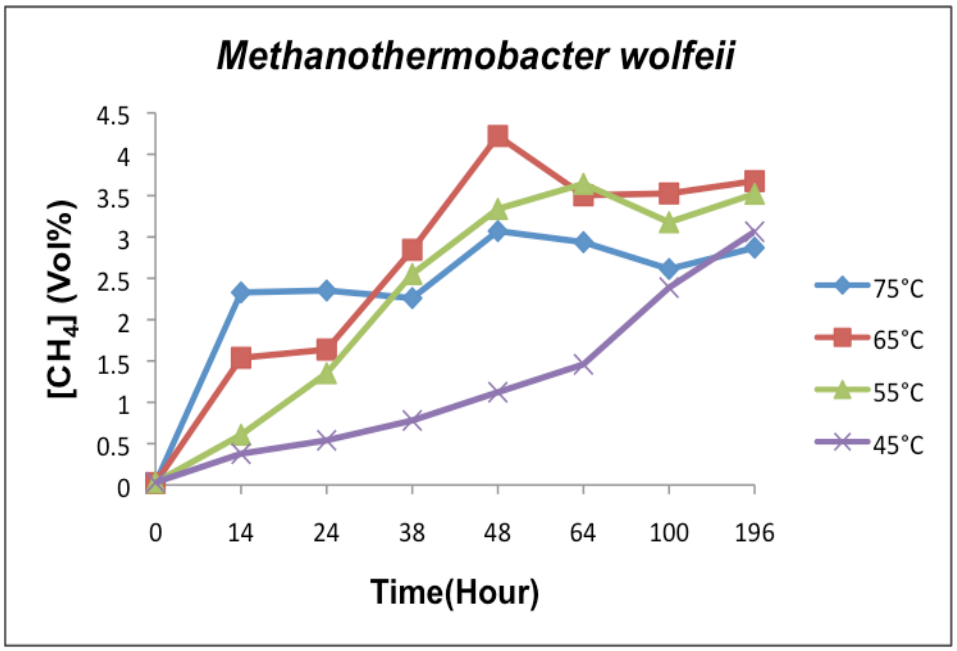

Figure 4.2. Methane concentration as a function of time at temperatures of 45ºC, 55ºC, 65ºC, and 75ºC in the absence of pressurized hydrogen gas.

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Chapter 5

Effects of Temperature and High Pressure on the Growth and Survivability of Methanogens and Stable Carbon Isotope Fractionation: Implications for Deep Subsurface Life on Mars

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5.1 Abstract

In order to examine the potential survivability of life in the Martian deep subsurface, we have investigated the effects of temperature (45ºC, 55ºC, and 65ºC) and pressure (1 atm, 400 atm, 800 atm, and 1200 atm) on the growth, carbon isotopic data, and morphology of chemolithoautotrophic anaerobic methanogenic archaea, *Methanothermobacter wolfeii*. The growth and survivability of this methanogen were determined by measuring the methane concentration in headspace gas samples after the cells were returned to their conventional growth conditions. Interestingly, this methanogen survived at all the temperatures and pressures tested. *M. wolfeii* demonstrated the highest methane concentration following exposure to pressure of 800 atm and a temperature of 65ºC. We found that the stable carbon isotopic fractionation of methane, $\delta^{13}C(CH_4)$, was slightly more enriched in ^{12}C at 1 atm and 55ºC than the carbon isotopic data obtained in other temperature and pressure conditions. A comparison of the images of the cells before and after the exposure to different temperatures and pressures did not show any obvious alteration in the morphology of *M. wolfeii*. The research reported here suggests that at least one methanogen, *M. wolfeii*, may be able to survive under hypothetical Martian subsurface conditions with respect to temperature and pressure.

5.2 Introduction

The current surface conditions of Mars are extremely harsh for any known life forms. Environmental factors such as low surface temperature, low atmospheric pressure, DNA damaging UV radiation, and the presence of oxidizing compounds make Mars an inhospitable planet (Biemann et al., 1977; Cockell et al., 2000; Jakosky, 1998). If life exists on the surface of Mars, it would have to challenge the environmental extremes of Mars and would have unique adaptation mechanisms. Therefore, the most feasible possibility of finding active known life forms on Mars would be "near or deep subsurface", where the temperature and pressure will be higher compared to the surface and the environment will be protected from the damaging cosmic radiation. The discovery of extremophiles and the knowledge of Earth's subsurface biospheres have also bolstered the idea of searching for life in the subsurface of other planetary bodies such as Mars (Cavicchioli, 2002).

The detection of methane in the Martian atmosphere (Formisano et al., 2004; Krasnopolsky et al., 2004; Mumma et al., 2004; Mumma et al., 2009; Webster et al., 2015) has further reinforced the search for extinct or extant life on Mars. The reason is that most terrestrial methane is produced by biological sources either directly or indirectly (Atreya et al., 2007). Methanogens are one of the various sources of methane on Earth. Some strains of methanogens have shown survivability and growth in simulated Martian physical and chemical conditions (Kral and Altheide, 2013; Kral et al., 2004; Kral et al., 2014; Kral et al., 2015; McAllister and Kral, 2006; Sinha and Kral, 2015). For these reasons, methanogens, which are chemolithoautrophic anaerobic archaea, have been considered ideal candidates for life on Mars (Boston et al., 1992; Chapelle et al., 2002; Chastain and Kral, 2012; Kral et al., 2004; Kral et al., 2015; Moran et al., 2005). The sources of methane on Mars are still unknown. Mars'

atmospheric methane could be the results of biotic, abiotic or a combination of both processes. Methanogens could be one of the several potential sources of Martian methane.

Stable carbon isotope fractionation is one of the several potential techniques to differentiate between biogenic and abiogenic sources of methane (Allen et al., 2006). Stable carbon isotope fractionation data for terrestrial atmospheric methane have been used in order to understand the environments, pathways, and origins or substrates of methanogenesis (Londry et al., 2008; Rothschild and DesMarais, 1989; Schidlowski, 1992). Sinha and Kral have recently studied the carbon isotope fractionation following methanogenesis on various Mars regolith analogs and found enriched values of ^{12}C on the clay called montmorillonite compared to the carbon isotopic data obtained on other Mars analogs such as JSC Mars-1, Artificial Mars Simulant, and Mojave Mars Simulant (Sinha and Kral, 2015).

Analogous to Earth's subsurface environments, hydrothermal systems might have existed and may also be present on Mars. On Earth, several hyperthermophilic and barophilic archaea have been isolated and characterized from the deep sea floor and hydrothermal sites (Canganella et al., 1997; Horikoshi, 1998; Shimizu et al., 2011; Takai et al., 2002). Some of the most abundant species near hydrothermal vents are hyperthermophilic methanogens (Takai et al., 2004). Life near a hydrothermal vent experiences a wide range of temperature and pressure. It has been found that a methanogen of genus *Methanopyrus* can grow up to a temperature of 110^{o}C (Takai et al., 2008) and *Methanococcus jannaschii* demonstrated methanogenesis up to a pressure of 750 atm (Miller et al., 1988). Methanogenesis has also been found in organisms thriving in 3.5-million-year-old subseafloor basalt on Earth and was detected by using $\delta^{13}C$ data (Lever et al., 2013). Several surface features, geochemical, and isotopic evidence in a Martian meteorite point to the activity of hydrothermal systems on Mars (Brakenridge et al., 1985;

Romanek et al., 1994; Shock, 1997; Watson et al., 1994). Therefore, similar subsurface biota might exist in the Mars' subsurface.

There are a few studies done on the effect of temperature and pressure on various strains of methanogens. However, the effects of a wide range of temperature and high pressure on methanogens in the context of Mars' subsurface have not been studied before. The goal of this study is to examine the growth and survivability of a Mars' model organism, *Methanothermobacter wolfeii* in a wide range of temperatures (45^{o}C - 65^{o}C) and pressures (1 atm, - 1200 atm). *M. wolfeii*, a hydrogenotrophic methanogenic archaeon used in this study utilizes CO_2 for its carbon source, H_2 for its energy source and produces methane as a metabolic byproduct. We have also measured the stable carbon isotope fractionation of methane, $\delta^{13}C(CH_4)$, in order to understand the effect of optimal and non-optimal temperature and pressure on the carbon isotopic data. The images of cells were also acquired and were analyzed to investigate morphological changes following exposure to various pressures and temperatures.

5.3 Materials and Methods

5.3.1 Preparation of a Stock Culture of *M. wolfeii*

M. wolfeii (OCM36) was obtained from the Oregon Collection of Methanogens, Portland State University, Portland, OR and was grown in MM medium consisting of potassium phosphate, ammonium chloride, calcium chloride, resazurin as an oxygen indicator, and many trace minerals (Xun et al., 1988) in a bicarbonate buffer. MM medium was prepared in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI), which was filled with 90% carbon dioxide and 10% hydrogen. The growth medium was then transferred into anaerobic culture tubes inside the anaerobic chamber as described previously

((Boone et al., 1989). The tubes were then sealed with butyl rubber stoppers, removed from the chamber, crimped with aluminum caps, and autoclaved for sterilization.

A sterile sodium sulfide solution (2.5% wt/vol; 0.15 mL per 10 mL media) was then added to each tube about an hour prior to inoculation of the methanogen (Boone et al., 1989) in order to eliminate any residual molecular oxygen from the tubes containing the media. After inoculating the methanogen, the tubes were pressurized with 2 atm of H_2 gas and incubated at the organism's optimal growth temperature, 55^{o}C. The stock culture of *M. wolfeii* was maintained by transferring into fresh MM medium every fifteen days.

The growth and survivability of this methanogen were determined by measuring the methane concentration in an aliquot of the headspace gas using a gas chromatograph (Shimadzu 2014, USA), which was equipped with a flame ionization detector.

5.3.2 Temperature-Pressure Experiments

The schematic diagram of the temperature-pressure chamber is shown in Figure 5.1. A quartz cuvette (Spectracell, Oreland, PA) was filled with 1 mL of fresh liquid culture of *M. wolfeii* and was capped with a Teflon cap (E. I. DuPont de Nemours, Paris, France) inside the anaerobic chamber. The cuvette filled with sample was then put into a high hydrostatic pressure-temperature chamber (ISS, Champaign, IL), which was filled with water and was connected to a piston. The pressure inside the chamber was developed with the help of a piston by pressurizing liquid water, and the pressure was measured with a pressure gauge attached to the piston (Kumar and Libchaber, 2013). The temperature of the chamber was maintained using a circulating water bath (Neslab, USA) and was measured in real time using a thermocouple (National Instruments, USA) attached to the chamber. Before loading the sample, the temperature of the chamber was

equilibrated to the desired temperature and the pressure was applied after the sample cuvette was put into the high temperature-pressure chamber. The system equilibrated to the desired pressure and temperature in less than two minutes after loading the sample. In this work, we preformed experiments at three different temperatures—45^{o}C, 55^{o}C, and 65^{o}C and four different pressures—1 atm, 400 atm, 800 atm, and 1200 atm; resulting in a total of twelve sets of experiments. For a given temperature and pressure, the sample was kept in the temperature-pressure chamber for 15 hours. After this time, the pressure of the chamber was released and the sample-filled cuvette was removed from the chamber.

The cells from the cuvette were then transferred to fresh medium. Five hundred microliters of cells from the cuvette were mixed with 500 uL of sterilized MM medium in a vial to make a total of 1 mL of culture. From this, 300 uL cells were inoculated into three different anaerobic tubes containing 10 mL of sterilized MM medium to make triplicate samples. The tubes were then pressurized with 2 atm H_2 gas and incubated at the conventional growth temperature, 55^{o}C. The growth and survivability were determined by measuring methane concentration in the headspace gas of each sample at regular intervals with the help of a gas chromatograph (Shimadzu 2014).

5.3.3 Determination of Stable Carbon Isotope Fractionation

Using the procedure described by Sinha and Kral (2015), the carbon isotope fractionation of methane in the headspace gas of all samples was measured periodically by a Piccaro Cavity Ringdown Spectrometer G-2201-I isotopic CO_2/CH_4 in the University of Arkansas Isotope lab. The carbon isotope fractionation, $\delta^{13}C$, was calculated using the following equation:

$$\delta^{13}C_{Sample} = \left\{ \frac{\left(\frac{^{13}C}{^{12}C}\right)_{Sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{Reference}} - 1 \right\} * 1000$$

The reference isotopic standard for $\delta^{13}C$ is Pee Dee Belemonite (O'Leary, 1981).

5.3.4 Imaging of Cells:

Phase contrast images of the cells were obtained before and after exposure to various temperatures and pressures using a SPOT Imaging camera and 40X objective mounted on a Nikon optiphot microscope.

5.4 Results

M. wolfeii was exposed to temperatures of 45ºC, 55ºC, and 65ºC and pressures of 1 atm, 400 atm, 800 atm, and 1200 atm in a high hydrostatic pressure-temperature chamber for 15 hours. Interestingly, *M. wolfeii* survived at all temperatures and pressures studied here. This methanogenic archaeon demonstrated methanogenesis by producing methane after the cells were returned to their conventional growth conditions. All measurements of methane concentration were taken post exposure to different temperatures and pressures.

In Figure 5.2, we show methane concentration, [CH_4], as a function of time for the cells exposed to the temperatures—45ºC, 55ºC, and 65ºC. Data in these figures represent the average methane concentration produced by methanogens exposed to the pressures—1 atm, 400 atm, 800 atm, and 1200 atm. For all temperatures and pressures, samples were in triplicates. For each temperature, *M. wolfeii* demonstrated highest methane concentration following the exposure to 800 atm of pressure and the lowest methane concentration following 1 atm. Highest methane

concentration varies for different temperatures and reaches the maximum on different days. For example, at 800 atm and 45^{o}C and 55^{o}C, methane concentration reached its maximum on the third day while for 65^{o}C the methane concentration reached its maximum on the fourth day. Moreover, we found that at 55^{o}C, the optimal growth temperature of *M. wolfeii*, the lag phase was little more than 24 hours whereas at 45^{o}C and 65^{o}C (non-optimal growth temperatures), the lag phases were less than 24 hours. This suggests that after the release of non-optimal physical conditions (stress), *M. wolfeii* adapted quickly to the optimal growth condition.

In Figure 5.3, we show methane concentration, [CH_4], as a function of time for the cells exposed to the pressures—1 atm, 400 atm, 800 atm, and 1200 atm. Data in these figures represent methane produced by methanogens exposed to the temperatures—45^{o}C, 55^{o}C, and 65^{o}C. For all temperatures and pressures, samples were in triplicates. For each pressure, *M. wolfeii* exhibited the highest methane concentration after exposure to 65^{o}C. For the lowest pressure, 1 atm, the methane concentration reached its maximum on the third day following a temperature of 65^{o}C. However, for the highest pressure, 1200 atm, the methane concentration reached its maximum on the fourth day for all of the temperatures tested.

We next studied the effect of temperature and pressure on the stable carbon isotope fractionation of methane produced by *M. wolfeii*. In Table 5.1, we have listed the carbon isotope fractionation data obtained on the second and sixth day following the pressure-temperature exposures. For 1 atm and 55 ^{o}C (conventional growth conditions of *M. wolfeii*), $\delta^{13}C(CH_4)$ on the second and sixth day are -74.36‰ and -73.35‰, which are comparable to published data for standard growth conditions (Sinha and Kral, 2015). Here we found slightly depleted values of $\delta^{13}C(CH_4)$ in the conventional conditions compared to the $\delta^{13}C(CH_4)$ data obtained at non-optimal temperatures and elevated pressures.

In order to compare any morphological changes in the cells exposed to optimal and non-optimal pressures and temperature, we obtained phase contrast images of the cells before and after exposure to different temperature and pressure. In Figure 5.4, we compare the images of cells exposed to temperatures—55ºC and 65ºC, and pressures—1 atm and 800 atm. We did not find any significant effect of temperature and pressure on the morphology of *M. wolfeii.* It has been shown that high pressure exhibits cell division inhibiting effects on mesophilic bacteria such as *E. coli* (Kumar and Libchaber, 2013). The lack of morphological alteration such as elongation in the case of *M. wolfeii* suggests that the exposure time of 15 hours was not long enough for the cells to undergo multiple cycles of cell division.

5.5 Discussion

The growth and survivability of several methanogenic archaea in simulated Martian surface conditions such as at low temperature, low pressure, and desiccation have been studied previously (Kendrick and Kral, 2006; Kral et al., 2011; Kral et al., 1998; Reid et al., 2006). On the other hand deep subsurface of Mars could potentially offer a feasible environment for a biosphere. The challenges are then high pressure, temperature, and availability of liquid water, nutrients, and the source of energy. It is imperative to locate liquid water in the subsurface of Mars. All terrestrial life needs water at some stage in their life cycle. Martian geophysical models suggest that the liquid water in the subsurface of Mars could be present to a depth of ~310 km (Jones et al., 2011). According to this model, the depth and pressure for approximate temperature range 45ºC-65ºC would be between 1-30 km and 100-3,000 atm.

In this work, we have investigated the effects of temperature (45ºC, 55ºC, and 65ºC) and pressure (1 atm, 400 atm, 800 atm, and 1200 atm) on the growth, carbon isotopic data, and

morphology of *M. wolfeii*. The growth and survivability of methanogens were determined by measuring methane concentration in the headspace gas samples after they were returned to their conventional growth conditions. Due to the limitation on the maximum temperature and prolonged experiments in hydrostatic temperature-pressure chamber, 15 hours long experiments were performed with a maximum temperature of 65^{o}C and pressure of 1200atm. Since the typical doubling time for *M. wolfeii* is about 10 hours at conventional growth conditions, our experiments should able to detect the survivability over the time scale of our experiments. We found *M. wolfeii* was able to endure a temperature of 65^{o}C and a pressure of 1200 atm for at least that amount of time. Surprisingly, *M. wolfeii* demonstrated methanogenesis following exposure to all the temperatures and pressures studied here. *M. wolfeii* exhibited the highest methane concentration following exposure to a pressure of 800 atm and a temperature of 65^{o}C. The exact reason for this is not clear and will be the focus of future studies.

The stable carbon isotope fractionation of methane was measured in different temperature and pressure experiments. We found very slight or negligible difference in the carbon isotopic data following optimal and non-optimal growth conditions of *M. wolfeii.* The $\delta^{13}C(CH_4)$ data in the optimal conditions were slightly lower than the $\delta^{13}C(CH_4)$ data in non-optimal conditions. It is possible that for *M. wolfeii,* 15 hours exposure to various temperatures and pressures may not be long enough to have a significant effect on the carbon isotopic data. (Takai et al., 2008) have found that *Methanopyrus kandleri* produced isotopically heavier methane under high hydrostatic pressure conditions compared to the methane produced by *M. kandleri* in a conventional growth condition (Takai et al., 2008).

We compared the morphology of cells before and after the exposure to different temperatures and pressures. Most of the cells remained intact and we did not find obvious

alteration in the morphology of *M. wolfeii*, unlike *E. coli*, which shows increase in cell length with increase in pressure (Kumar and Libchaber, 2013). Since the doubling time of *E. coli* is very small as compared to *M. wolfeii*, it was possible to detect stochasticity in cell division and elongation of *E. coli* at high pressure over a large number of generation times.

The results presented here suggest that one Mars' model microorganism, *M. wolfeii*, can survive under presumed Martian subsurface conditions in terms of temperature and pressure. Therefore, the search for life on Mars should also be focused on the deep- subsurface of Mars.

5.6 Conclusions

Methanogens have been considered ideal life forms on Mars for a long time. Here, we have examined the growth and the survivability of a methanogen, *M. wolfeii*, in presumed deep-subsurface environments in terms of temperature and pressure. We used three different temperatures (45^{o}C, 55^{o}C, and 65^{o}C) and four different pressures (1 atm, 400 atm, 800 atm, and 1200 atm). *M. wolfeii* demonstrated survivability by producing methane following exposure to all different temperatures and pressures. The growth and survivability of *M. wolfeii* were investigated after returning them to their conventional growth conditions. We have also measured the carbon isotopic fractionation of methane produced by *M. wolfeii* and found that $\delta^{13}C(CH_4)$ in optimal growth conditions was slightly lower than the values obtained in non-optimal growth conditions. A comparison of the images of cells before and after the exposure to different temperatures and pressures did not reveal any apparent alteration in the morphology of *M. wolfeii*.

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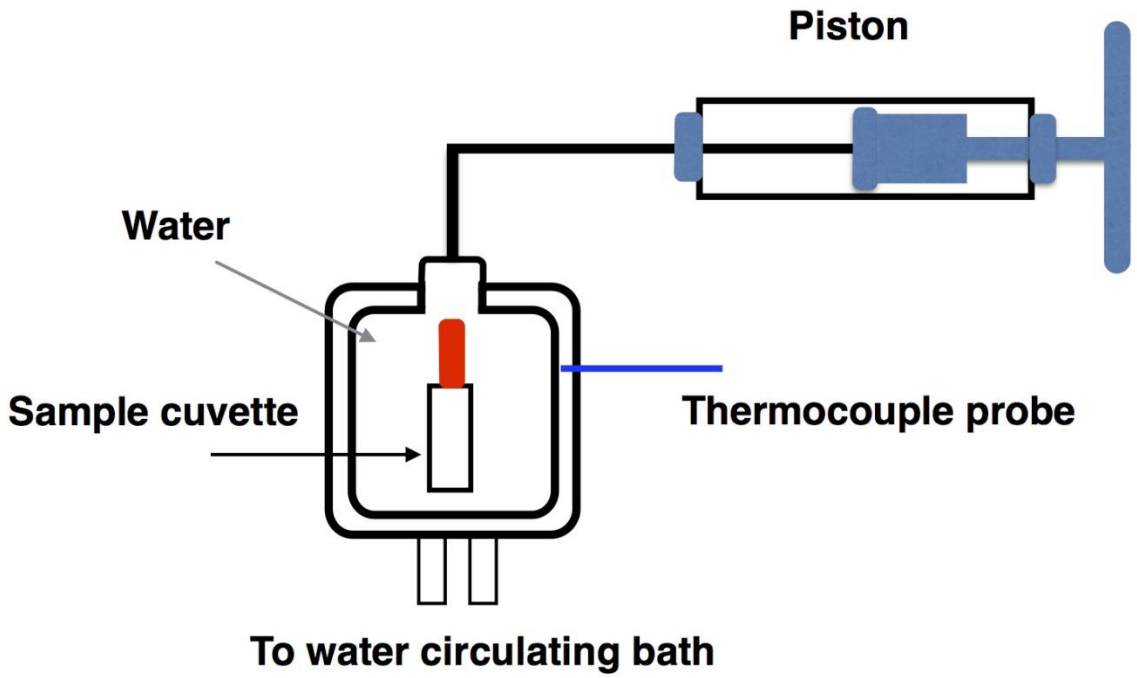

Figure 5.1: Experimental setup for the temperature-pressure experiment.

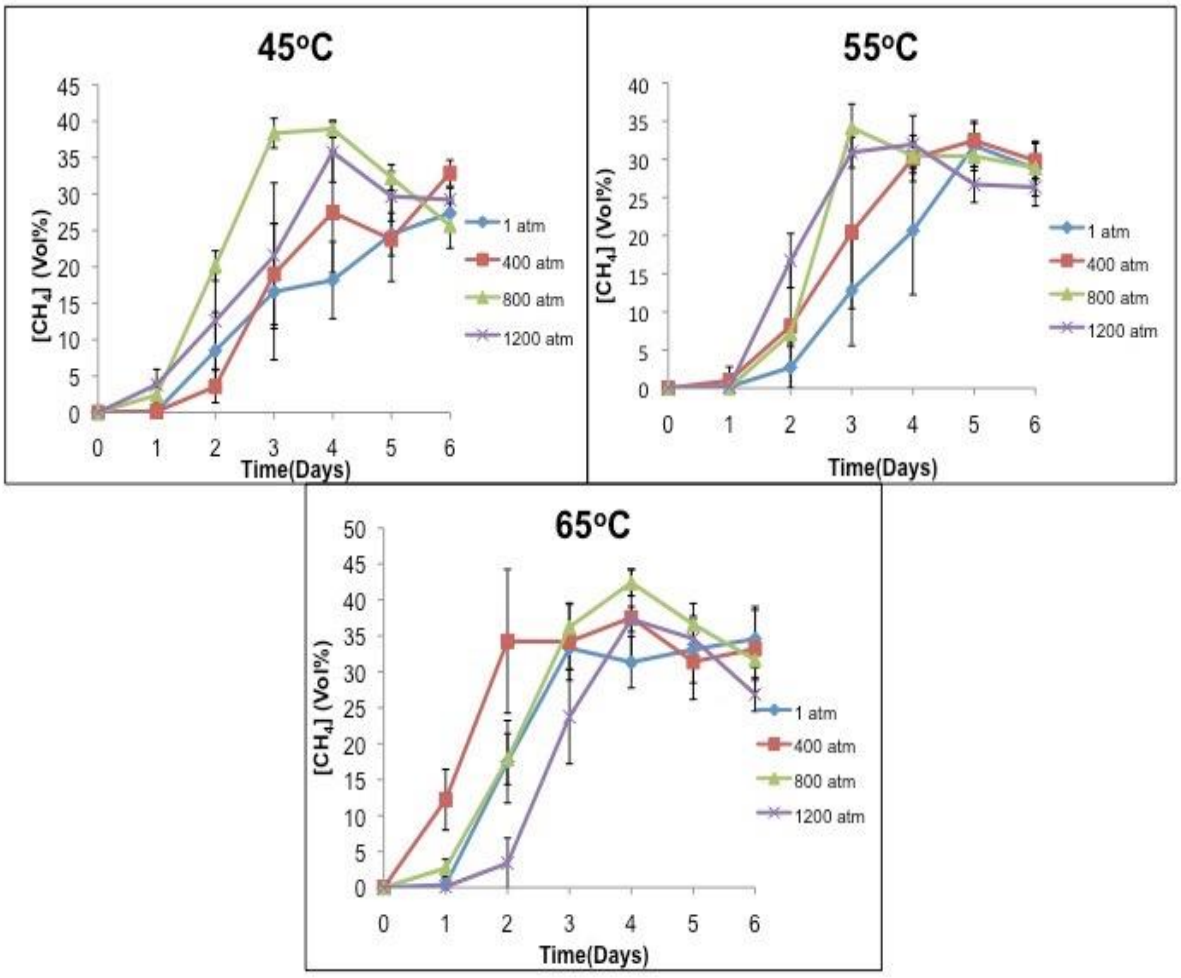

Figure 5.2: Methane concentration as a function of time following methanogenesis for *Methanothermobacter wolfeii* exposed to the temperatures—45°C, 55°C, and 65°C. The different colored lines in these figures represent the average methane concentration produced by methanogens exposed to the pressures—1 atm, 400 atm, 800 atm, and 1200 atm.

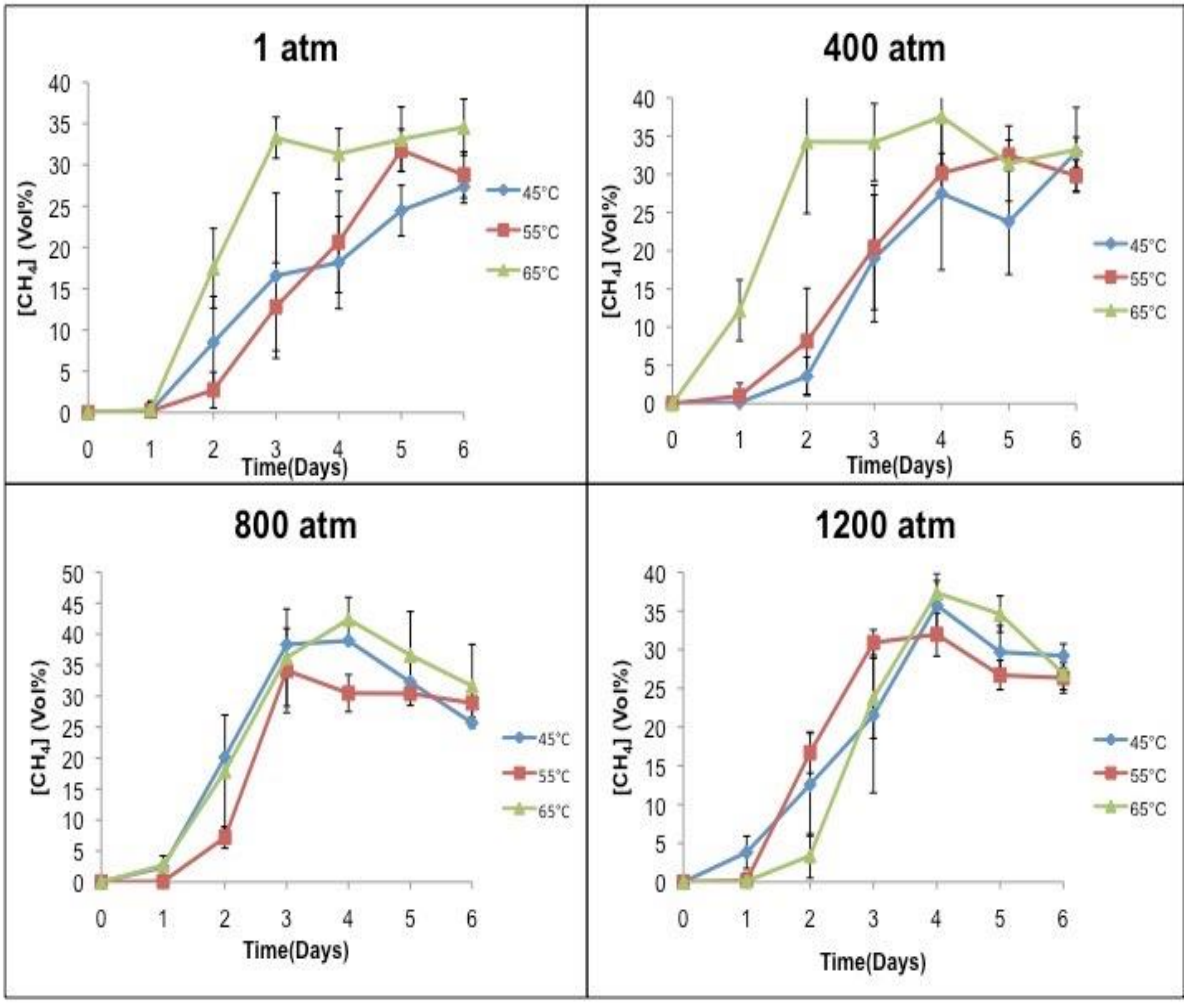

Figure 5.3: Methane concentration as a function of time following methanogenesis for *Methanothermobacter wolfeii* exposed to the pressures—1 atm, 400 atm, 800 atm, and 1200 atm. The different colored lines in these figures represent the average methane concentration produced by methanogens exposed to the temperatures—45ºC, 55ºC, and 65ºC.

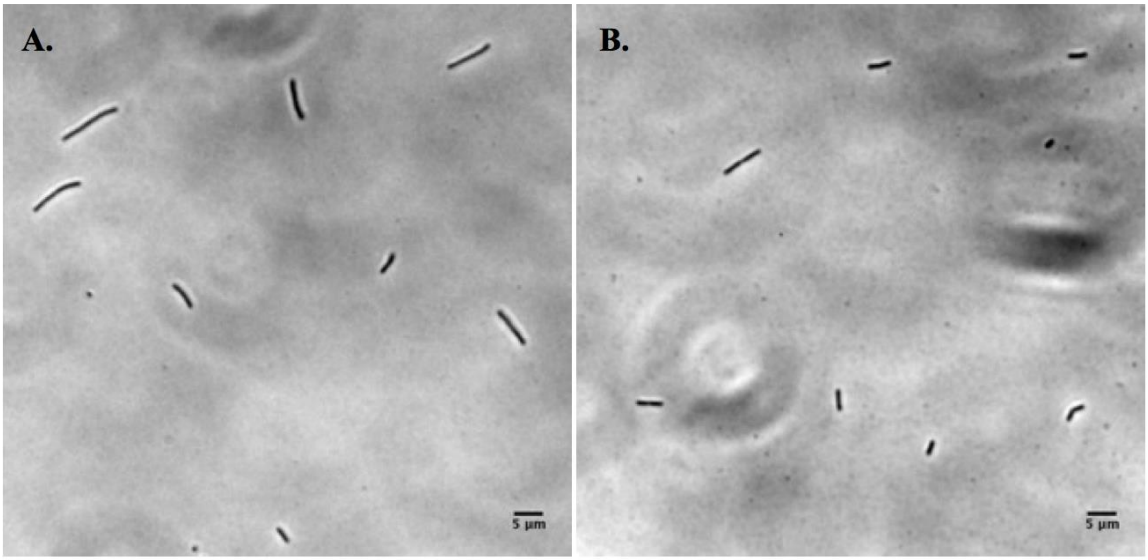

Figure 5.4: Images of *Methanothermobacter wolfeii* after exposure to (A) T=55ºC and P=1 atm (B) T=65ºC and P=1200 atm under a magnification of 400X.

Table 5.1: Carbon isotope fractionation of methane, $\delta^{13}C(CH_4)$, produced by *Methanothermobacter wolfeii* obtained on Day 2 and Day 6 following different temperature-pressure exposures. Values shown are in per mil.

	45°C		55°C		65°C	
Pressure (atm)	Day 2	Day 6	Day 2	Day 6	Day 2	Day 6
1	-70.86	-70.74	-74.36	-73.35	-70.43	-70.94
400	-72.30	-71.98	-70.96	-71.04	-69.38	-69.66
800	-72.88	-73.06	-73.25	-71.55	-71.42	-69.72
1200	-73.74	-69.79	-69.13	-70.64	-69.55	-69.48

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Chapter 6

Survivability and Growth Kinetics of Methanogenic Archaea at Various pHs and Pressures: Implications for Deep Subsurface Life on Mars

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6.1 Abstract

Life as we know it requires liquid water and sufficient liquid water is highly unlikely on the surface of present-day Mars. However, according to thermal models there is a possibility of liquid water in the deep subsurface of Mars. Thus, the martian subsurface, where the pressure and temperature is higher, could potentially provide a hospitable environment for a biosphere. Also, methane has been detected in the Mars' atmosphere. Analogous to Earth's atmospheric methane, martian methane could also be biological in origin. The carbon and energy sources for methanogenesis in the subsurface of Mars could be available by downwelling of atmospheric CO_2 into the regolith and water-rock reactions such as serpentinization, respectively. Corresponding analogs of the martian subsurface on Earth might be the active sites of serpentinization at depths where methanogenic thermophilic archaea are the dominant species. Methanogens residing in Earth's hydrothermal environments are usually exposed to a variety of physiological stresses including a wide range of pressures, temperatures, and pHs. Martian geochemical models imply that the pH of probable groundwater varies from 4.96 to 9.13. In this work, we used the thermophilic methanogen, *Methanothermobacter wolfeii*, which grows optimally at 55^{o}C. Therefore, a temperature of 55^{o}C was chosen for these experiments, possibly simulating Mars' subsurface temperature. A martian geophysical model suggests depth and pressure corresponding to a temperature of 55^{o}C would be between 1-30 km and 100-3,000 atm respectively. Here, we have simulated Mars deep subsurface pH, pressure, and temperature conditions and have investigated the survivability, growth rate, and morphology of *M. wolfeii* after exposure to a wide range of pH (5 to 9) and pressure (1 − 1200 atm) at a temperature of 55^{o}C. Interestingly, in this study we have found that *M. wolfeii* was able to survive at all the pressures and pHs tested at 55^{o}C.

In order to understand the effect of different pHs and pressures on the metabolic activities of *M. wolfeii*, we also calculated their growth rate by measuring methane concentration in the headspace gas samples at regular intervals. In acidic conditions, the growth rate (γ) *of M. wolfeii* increased with the

increase in pressure. In neutral and alkaline conditions, the growth rate (γ) *of M. wolfeii* initially increased with pressure, but decreased upon further increase of pressure. To investigate the effect of combined pH, pressure, and temperature on the morphology of *M. wolfeii*, we took phase contrast images of the cells. We did not find any obvious significant alteration in the morphology of *M. wolfeii* cells. Methanogens, chemolithoautotrophic anaerobic microorganisms, are considered as ideal model microorganisms for Mars. In light of research presented here, we suggest that at least one methanogen, *M. wolfeii*, could survive in the deep subsurface environment of Mars.

6.2 Introduction

Mars is an inhospitable planet because of the low atmospheric surface pressure (as low as 7 mbar), low surface temperature (average of 210K), intense DNA damaging radiation fluxes (Cockell et al., 2000), and the presence of oxidizing compounds in the top soil (Biemann et al., 1977). However, Martian deep subsurface could provide hospitable environments for a biosphere where pressure and temperature would be higher (Jones et al., 2011), liquid water could be present (Malin and Edgett, 2000), and the surroundings are protected from cosmic radiation. Furthermore, our knowledge of subsurface terrestrial life on Earth lends support to the possibility of biological communities in the subsurface of Mars.

On Earth, where there is liquid water on the surface and in the subsurface, there is life irrespective of the harsh ambient physical and chemical conditions. Despite its inhospitable environment today, Mars has a rich history of aqueous processes. Indeed, a recent study of Mars, the spectral evidence suggests the presence of hydrated salt in the recurring slope lineae (RSL), and these RSLs could be the result of contemporary surface/subsurface activity of melting water (Ojha et al., 2015). Additionally, clays and carbonates have been found in one of the deepest craters of Mars, the McLaughlin crater, and it has been hypothesized that these minerals could have formed in an alkaline and groundwater environment (Michalski et al., 2013). The stable isotopic ratios of oxygen and hydrogen in the Martian atmosphere indicate that a substantial amount of water has been lost to space (Webster, 2013; Mahaffy, 2013; Owen et al., 1988). These geological evidences thus support a warmer and wetter Mars early in its history (Baker, 2001; Malin and Edgett, 2000; Carr M.H., 1996). However, mathematical models imply that a substantial amount of water still remains today from the ancient era and exists as a near surface cryosphere and as ground water residing deeper in the crust (Clifford et al., 2010). A few subsurface conditions such as radiogenic heating, the pressure above the triple point of water, and the presence of

dissolved salts could allow for Martian ground water in the cryosphere (Weiss et al., 2000; Clifford et al., 2010). A Martian pressure-temperature model for a potential Martian biosphere suggests that liquid water could be available up to 310 km below the surface of Mars (Jones et al., 2011). Moreover, surface features on Mars such as channels, chaotic and fretted terrains, and some rifted basins (chasmata) are most probably formed by ancient hydrothermal systems (Farmer, 1996).

To contemplate subsurface biota on Mars, it is important to establish sources of energy and carbon in Martian subsurface environments in addition to water. Based on geochemical and thermodynamic considerations, it has been suggested that geologically derived hydrogen could be the primary energy source (Chapelle et al., 2002). The interaction of hydrothermal fluid with reduced basalt can produce H_2 through serpentinization (Lyons et al., 2005; Oze and Sharma, 2005; Schulte et al., 2006). Furthermore, the discovery of serpentine minerals from the orbiter in the terrains and craters of Mars suggests present or past serpentinization processes inside the planet (Ehlmann et al., 2010). The carbon could be derived from one or more sources such as dissolved CO_2 in the ground water, diffusion of atmospheric CO_2 into the regolith (Weiss et al., 2000), and sequestration of CO_2 in carbonates (Farmer et al., 1996; Varnes et al. 2003; Kral et al., 2014). The geochemical models for Martian hydrothermal systems also suggest ample amounts of chemical energy may be available for putative Martian microorganisms if the composition of host rock is the same as the composition of Martian meteorites (Varnes et al., 2003). All these pieces of evidence point to the possibility of Martian subsurface biota.

The pressure, temperature, and pH are the important factors for physiological activities of life. On Earth, the hydrostatic and lithostatic pressures increase at a rate of 0.10 atm (10.5 KPa) and 0.22 atm (22.6 KPa) per meter, respectively (Rothschild and Mancinelli, 2001). Terrestrial extremophiles such as barophiles, hyperthermophiles, acidophiles, and alkaliphiles have been proliferating in the subsurface of Earth, including hydrothermal systems (Cavicchioli, 2002; Jones et al., 1998; Karl, 1995;

Schleper et al., 1995). Geophysical models of Mars suggest that the approximate depth and pressure corresponding to a temperature 55°C would be between 1-30 km and 100-3,000 atm, respectively (Jones et al., 2011). Martian geochemical models suggest that the pH of probable groundwaters varies from 4.96 to 9.13 and the pH of plausible hydrothermal fluid ranges from 5.94 to 8.23 (Varnes et al., 2003). Moreover, the deep subsurface of Mars could have alkaline and anoxic environments because of the reactions of fluids with ultramafic rocks, analogous to Earth.

Terrestrial microorganisms that grow in extreme environments mostly belong to the domain Archaea because archaeal cytoplasmic membranes have unique membrane lipid bonding, distinct from bacterial and eukaryal cytoplasmic membrane lipids. In order to survive in thermophilic and barophilic environments, microbial cell membrane crystalline fluidity must be maintained (Kaneshiro, 1995). To survive in acidic environments, microorganisms must maintain pH homeostasis. Archaeal cell envelopes have higher stability, more rigidity, and less permeability to protons and can withstand a wide range of pH, pressure, and temperature. Therefore, archaea are the most suitable candidates for putative life on Mars.

The discovery of methane in the Martian atmosphere ((Formisano et al., 2004; Krasnopolsky et al., 2004; Mumma et al., 2004; Mumma et al., 2009; Webster et. al., 2015) has further bolstered the plausibility of life on Mars as most of Earth's atmospheric methane has biological origins. A number of potential sources of methane on Mars, from the surface to the subsurface including methane producing archaea, such as methanogens, have been discussed (Atreya et al., 2007). The methane in Mars' atmosphere would need to be replenished continuously considering its short lifetime, which is about 300 years (Hunten, 1979). The carbon isotope fractionation of methane can potentially discriminate sources, pathways, and environments of methane on Mars (Allen et al., 2006; Atreya et al., 2007; Sinha and

Kral, 2015). A computational model suggests that the diffusion of methane from the potential sources at a depth to the atmosphere through the regolith column may take several hundred thousand Mars-years (Steven et al. 2015).

On Earth, the most dominant microbial community in serpentinization driven hydrothermal waters is hydrogen-consuming, methane-producing archaea (Chapell, et al. 2002; Mehay et al., 2013). Analogous to Earth's subsurface biosphere microbial community, chemolithoautotrophic microorganisms would be the most appropriate models for plausible microorganisms in the subsurface of Mars (Fish and Giovanni, 1999, Mancinelli, 2000). Thermophilic archaea residing in Earth's hydrothermal environments are usually exposed to a variety of physiological stresses including a wide range of pressures, temperatures, and pHs (Lloyd et al., 2005; Miller et al., 1988).

Methanogens are chemolithoautotrophic anaerobic archaea, which mostly consume CO_2 as a carbon source and H_2 as an energy source and produce CH_4 as their metabolic byproduct. Methanogens have been considered ideal candidates for life forms on Mars for a long time (Boston et al., 1992; Chapelle et al., 2002; Chastain and Kral, 2012; Chastain and Kral, 2010a, b; Kendrick and Kral, 2006; Kral and Altheide, 2013; Kral et al., 2011; Kral et al., 2004; Kral et al., 1998; Kral et al., 2015; McAllister and Kral, 2006; Moran et al., 2005; Ormond and Kral, 2006; Sinha and Kral, 2015; Ulrich et al., 2010; Weiss et al., 2000). Therefore, it is important to understand the survivability and growth of Mars model microorganisms, methanogens, in a broad range of pH and high pressures simulating subsurface conditions of Mars. In the research reported here, we have examined the survivability, growth rate, and the morphology of a thermophilic methanogenic archaea, *Methanothermobacter wolfeii*, after exposure to a wide range of pressure (1, 400, 800, and 1200 atm) and pH (5 to 9 at intervals of 1) at their optimal growth temperature, 55^{o}C.

6.3 Materials and Methods

6.3.1 Preparation of Minimal Media (MM)

One hundred milliliters of MM medium consisting of potassium phosphate, ammonium chloride, calcium chloride, resazurin as an oxygen indicator, and many trace minerals (Xun et al., 1988) in a bicarbonate buffer were prepared in each of five separate flasks; one for neutral pH, two for acidic pH, and another two for alkaline pH. To make acidic MM medium, a flask of neutral pH medium was set on a hot plate. When the medium temperature reached 55^{o}C, 1N HCL was added drop by drop to the flask with continuous stirring until the pH of the medium became 5. Similarly, using another flask of neutral pH medium, pH 6 MM medium was also made. To make alkaline MM medium, two flasks of neutral pH medium were set on the hot plate one after another. At 55^{o}C, 1 M NaOH was added drop by drop to these flasks with continuous stirring until the pH of the medium reached the desired pH levels of pH 8 and 9.

All five flasks were left in a Coy Laboratories anaerobic chamber (Coy Laboratory Products Inc., Grass Lake Charter Township, MI) overnight for deoxygenation. The anaerobic chamber was filled with 90% carbon dioxide and 10% hydrogen. All growth media were then transferred into anaerobic culture tubes (10 mL of media in each tube) inside the anaerobic chamber as described by Boone et al. (1989). The tubes were then sealed with butyl rubber stoppers, removed from the chamber, crimped with aluminum caps, and autoclaved for sterilization.

6.3.2 Preparation of a Stock Culture of *Methanothermobacter wolfeii*

M. wolfeii (OCM36) was originally obtained from the Oregon Collection of Methanogens, Portland State University, Portland, OR and was grown in the neutral pH MM

medium. The stock culture was maintained by transferring into fresh MM medium every fifteen days.

6.3.3 Inoculation of Methanogens into Neutral, Acidic, and Alkaline pH MM Media

A sterile sodium sulfide solution (2.5% wt/vol; 0.15mL per 10 mL of media) was added to the MM media tubes about an hour prior to inoculation of the methanogens (Boone et al., 1989) in order to eliminate any residual molecular oxygen from the tubes. One half milliliter from the *M. wolfeii* stock was inoculated into each of two tubes of media with different pHs. After inoculating the methanogens, the tubes were pressurized with 2 atm of hydrogen gas and incubated at their optimal growth temperature, 55^{o}C. Methane concentration of the headspace gas of each sample was measured periodically using a gas chromatograph (Shimadzu 2014, USA).

6.3.4 pH-Pressure Experimental Setup

The experimental setup of the pressure-temperature chamber is shown in Figure 6.1. One milliliter of fresh liquid culture (in exponential phase) of *M. wolfeii*, either from acidic, neutral or alkaline stock, was transferred into a quartz cuvette (volume 1ml) (Spectracell, Oreland, PA) in the anaerobic chamber (Coy). The cuvette was then capped with a deformable Teflon cap (volume 200uL) containing 200uL of culture (E. I. DuPont de Nemours, Paris, France) inside the anaerobic chamber so that there was no liquid-air interface left inside the cuvette-teflon system. The deformable teflon cap allows for the required volume changes of the sample with pressure and temperature and hence, maintains the equilibrium pressure of the sample inside the cuvette with the external pressure of the pressurizing fluid (water). The capped cuvette was then placed into a high hydrostatic pressure-

temperature chamber (ISS, Champaign, IL) and filled with water. A high-pressure piston was then used to pressurize the pressurizing fluid (water), and the pressure was measured with a pressure gauge attached to the piston (Kumar and Libchaber, 2013). In this work, we used four different pressures–1 atm, 400 atm, 800 atm, and 1200 atm. A circulating water bath (Neslab, USA) connected to the high pressure-temperature chamber was used to control the temperature of the chamber and the temperature of the sample was measured in real time using a thermocouple (National Instruments, USA) connected to the pressure-temperature chamber. For a given pressure at 55^{o}C, the sample was kept in the high pressure-temperature chamber for 15 hours. The entire closed pressure-temperature chamber allowed the sample to remain anaerobic throughout the experiment.

After exposure to the desired pressure-temperature, 500µL of cells from the cuvette were then transferred into a vial containing 500µL of neutral MM medium. From this tube, 300µL of sample were inoculated into three different anaerobic tubes containing 10 mL of sterilized neutral pH MM medium containing sodium sulfide as described. These anaerobic tubes were then pressurized with 2 atm hydrogen gas and incubated at their optimum growth temperature of 55^{o}C. For statistical analysis of data, our samples were in duplicates and triplicates. A gas chromatograph (Shimadzu 2014, USA) was used to measure the methane concentrations of the headspace gas of each of the samples at regular intervals to assess the growth and survival of *M. wolfeii* after the exposure to different high pressures in optimal and non-optimal pH conditions. Phase contrast images of the cells were also acquired using a Nikon microscope with a 40X objective just after the exposure of *M. wolfeii* at varying pH and pressure at a temperature of 55^{o}C.

6.4 Results

A total of twenty sets of experiments were performed for five different pH values, 5, 6, 7, 8, and 9– and four different pressures– 1, 400, 800, and 1200 atm – at a temperature of 55^{o}C. To investigate if the exposure to combined pH-pressure-temperature changed the growth kinetics of cells, we measured methane concentration produced by *M. wolfeii* at regular intervals. For these experiments, cells were first exposed to different pH and pressure conditions for 15 hours in a pressure temperature chamber and then subsequently taken out of the chamber and re-inoculated in the neutral pH growth medium, pressurized with 2 atm hydrogen gas, and incubated at their optimum growth temperature of 55^{o}C. In Figures 6.2 through 6.6, we show methane concentration, [CH_4], as a function of time on a linear-log plot for the cells exposed to different pH levels and various pressures at 55^{o}C. For each pressure and pH, a set of duplicates/triplicates is shown.

We next calculated the growth rate of cells after exposure to different pressures and pHs. Concentration of CH_4 produced by cells at a given time t in the exponential growth phase can be written as:

$$\mathrm{CH_4(t)} = \int_0^{\mathrm{t}} \mathrm{N_0 e^{\gamma t'} dt'} + \mathrm{C} = \frac{\mathrm{N_0}}{\gamma}\left[\mathrm{e^{\gamma t} - 1}\right] + \mathrm{C} \qquad \text{Equation 1}$$

Where N_0 is the number of cells in the beginning of the exponential growth phase, γ is the growth rate of cells, and C is the concentration of methane at the beginning of exponential phase. γ is equal to $0.693/\tau$, where τ is the average doubling time of the cells. In Figures 6.2 through 6.6, we show the fit using Equation 1 (dotted lines) through the experimental data points. The average growth rate was extracted by fitting Equation 1 though the time-dependent

data of [CH_4] and averaging over the duplicates/triplicates.

In Figure 6.7, we show the average growth rate, γ, of the cells for different pH levels and various pressures. In the case of pH 5 (Figure 6.7A), we find that γ did not change significantly until 800 atm but suddenly increased at the highest pressure, 1200 atm. In the case of pH 6 (Figure 6.7B), we find that γ increased with the increase in pressure. In the case of pH 7 (Figure 6.7C), interestingly, we find that γ first increased with pressure but decreased from pressure 800 to 1200 atm. In alkaline conditions, (Figures 6.7D and 6.7E), we find a similar trend for γ; it first increased with pressure but decreased upon further increase of pressure. The pressure corresponding to the maximum in γ decreased upon increasing pH. A combined surface plot of pressure and pH dependence of growth rate is shown in Figure 6.8.

Past experiments on a mesophilic bacterium, Escherichia coli, have shown that high pressure affects cell division in bacteria (Kumar and Libchaber, 2013). Therefore, to see the effect of combined pH-pressure-temperature on the morphology of *M. wolfeii*, images of cells were also analyzed. However, we did not observe any obvious significant variations in their morphology. Representative phase contrast images of the cells at the lowest pressure (1 atm) and the highest pressure (1200 atm) at pH=7 are shown in Figure 6.9. Specifically, exposure to high pressure inhibits cell division and, hence, leads to elongation of cells. For cells to show any significant elongation as seen in the case of mesophilic bacteria, the time over which they are exposed to higher pressure must be longer than the doubling time of the cells. The typical doubling time of *M. wolfeii* is about 10 hrs. Hence the lack of changes in morphology, even at the highest pressure studied here, suggests that either the exposure of 15 hrs was not long enough for the cells to exhibit any morphological variations or *M. wolfeii* cell-division machinery is adapted to high pressures.

6.5 Discussion

We have examined the survivability of *M. wolfeii* in a broad range of pH– 5, 6, 7, 8, and 9– and pressures–1, 400, 800, and 1200 atm– at a temperature of 55^{o}C. Also, we have investigated the growth rate of *M. wolfeii* after they were brought back into their normal growth conditions: neutral pH medium, temperature 55°C and pressurized with 2 atm pressure of H_2. Interestingly, in all of the experiments, *M. wolfeii* exhibited survivability demonstrated by increasing methane production. This suggests that this specific methanogenic archaea could have repair/adaptation mechanisms to cope with stresses such as non-optimal pressures and pHs.

In order to understand the effect of various pHs and pressures on the metabolic functions of *M. wolfeii,* we calculated the growth rate of *M. wolfeii* by measuring methane concentration in headspace gas samples at regular intervals. In the acidic conditions, the growth rate (γ) of *M. wolfeii* increased following the increase in pressure. In the neutral and alkaline conditions, the growth rate (γ) of *M. wolfeii* reached a maximum following increasing pressure but decreased upon further increase of pressure. Earlier experimental studies have also demonstrated that the growth rate of thermophilic methanogenic archaea increased with the increase in the pressure up to 750 atm without increasing the optimum growth temperature of methanogens. (Bernhardt et al., 1988; Miller et al., 1988).

A comprehensive geophysical model of Mars found the approximate depth and pressure corresponding to our experimental temperature of 55°C to be between 1-30 km and 100-3,000 atm, respectively (Jones et al., 2011). Other workers used a simple heat diffusion model to suggest a shallower range between 2 and 7 km, yielding pressures of 220 and 730 km (Montgomery et al., 2009). In this research, we simulate Mars deep subsurface pressure conditions and exposed methanogens to a broad range of pressure from 1 to 1200 atm. Additionally, we attempted to simulate Mars geochemical conditions and utilized a pH range of media between 5 and 9. This pH

range is very close to the pH range of Martian groundwaters as suggested by the geochemical model of Varnes et al., 2003.

Next, we studied the morphology of *M. wolfeii* to examine the effect of pH and pressure on the integrity of cells. Previous studies (Bernhardt et al., 1988; Kumar and Libchaber, 2013) have shown that cell length increases with increasing pressure. In this work, we analyzed images acquired before and after exposure to various pHs and pressures. We did not find variability in the cell length of *M. wolfeii* suggesting 15 hours exposure time is not enough to see any alteration in their morphology.

The growth and survivability of microorganisms depend on their ambient physical and chemical conditions. The pH, pressure, and temperature not only affect the structural properties of biomolecules but also affect the biochemical reactions of a cell. On Earth, in geothermal environments, microorganisms experience a wide range of physiological stresses indicating microbes have environmental adaptation mechanisms. A large body of literature is available on the behavior of microorganisms in extreme physical and chemical conditions. However, effects of several pH levels, high pressure and temperature on *M. wolfeii* had not been studied previously. Further experiments are required in order to discuss *M. wolfeii* adaptation mechanisms under a wide range of pHs, pressures, and temperatures, including a complete understanding of its genome.

The results presented here demonstrate growth and survivability of the methanogenic archaea, *M. wolfeii*, following exposure to conditions presumed to occur in the deep subsurface environment of Mars. Any subsurface Martian life form, if present, would have to experience a wide range of pH, pressure and temperature. Even though we did not consider all stresses present in the deep subsurface of Mars, we have made an effort to understand physiological activities of methanogens under certain stress conditions. Analogous to Earth's subsurface, mineral rich hydrothermal systems could be favorable

environments for thermophilic anaerobic methanogenic archaea on Mars. Future work will examine the effects of broad ranges of temperatures on the growth and survivability of methanogens under elevated pressure conditions.

6.6 Conclusions

We have examined the survivability, growth rate, and morphology of *M. wolfeii* after exposure to a wide range of pH (5 to 9) and pressures (1-1200 atm) at temperature 55^{o}C. In this work, we used presumed pHs of groundwaters, and presumed pressures, and temperatures in the deep subsurface of Mars. *M. wolfeii* was able to tolerate all pHs and pressures tested at 55^{o}C. Following acidic conditions, the growth rate (γ) of *M. wolfeii* increased with increase in pressure. Following neutral and alkaline conditions, the growth rate (γ) of *M. wolfeii* initially increased following increases in pressure, but decreased upon further increase of pressure. In order to investigate the combined effect of pH-pressure-temperature on the morphology of *M. wolfeii*, we examined images of the cells after exposure to different pHs and pressures. We did not find any noticeable changes in the morphology of *M. wolfeii.* Based on the results presented here, we suggest that at least one type of methanogenic archaea, *M. wolfeii* could possibly survive and thrive in the deep subsurface conditions of Mars.

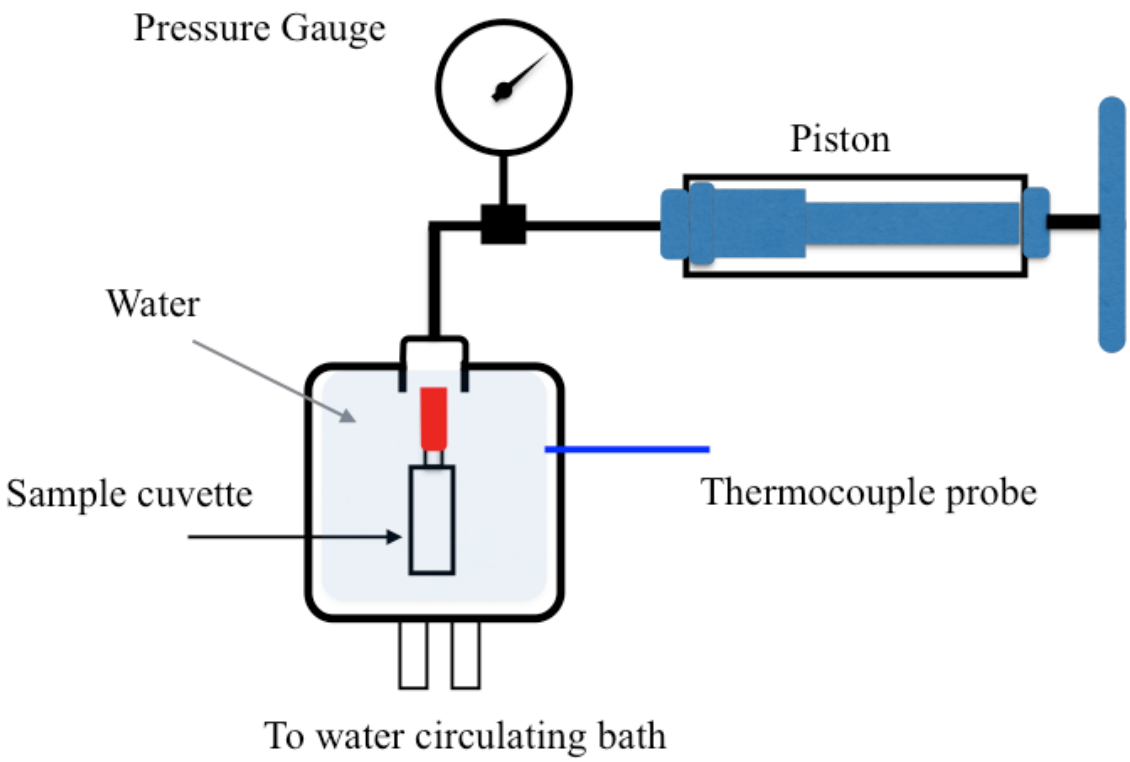

Figure 6. 1: Schematic of high pressure-temperature setup.

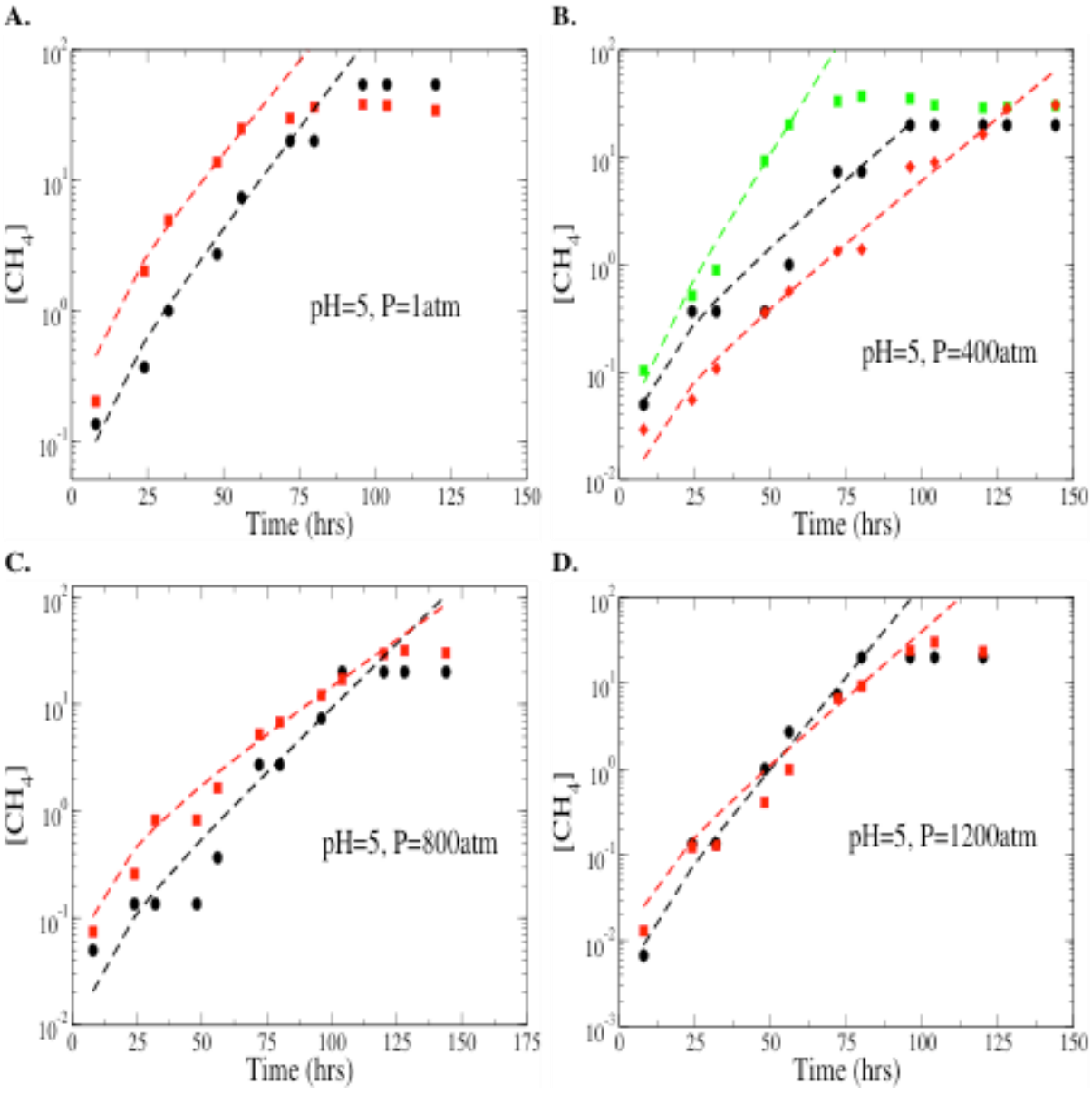

Figure 6.2: Growth curves represented by methane concentration produced by *Methanothermobacter wolfeii* as a function of time; Linear-log plot of methane production measured after exposure to pressures (A) P=1 atm (B) P=400 atm (C) P=800 atm (D) P=1200 atm, at pH=5 and temperature 55ºC. Symbols are the experimental data and color lines represent the fit using Eq. 1 through the data points in the exponential regime. Duplicates/triplicates are shown for all the pressures.

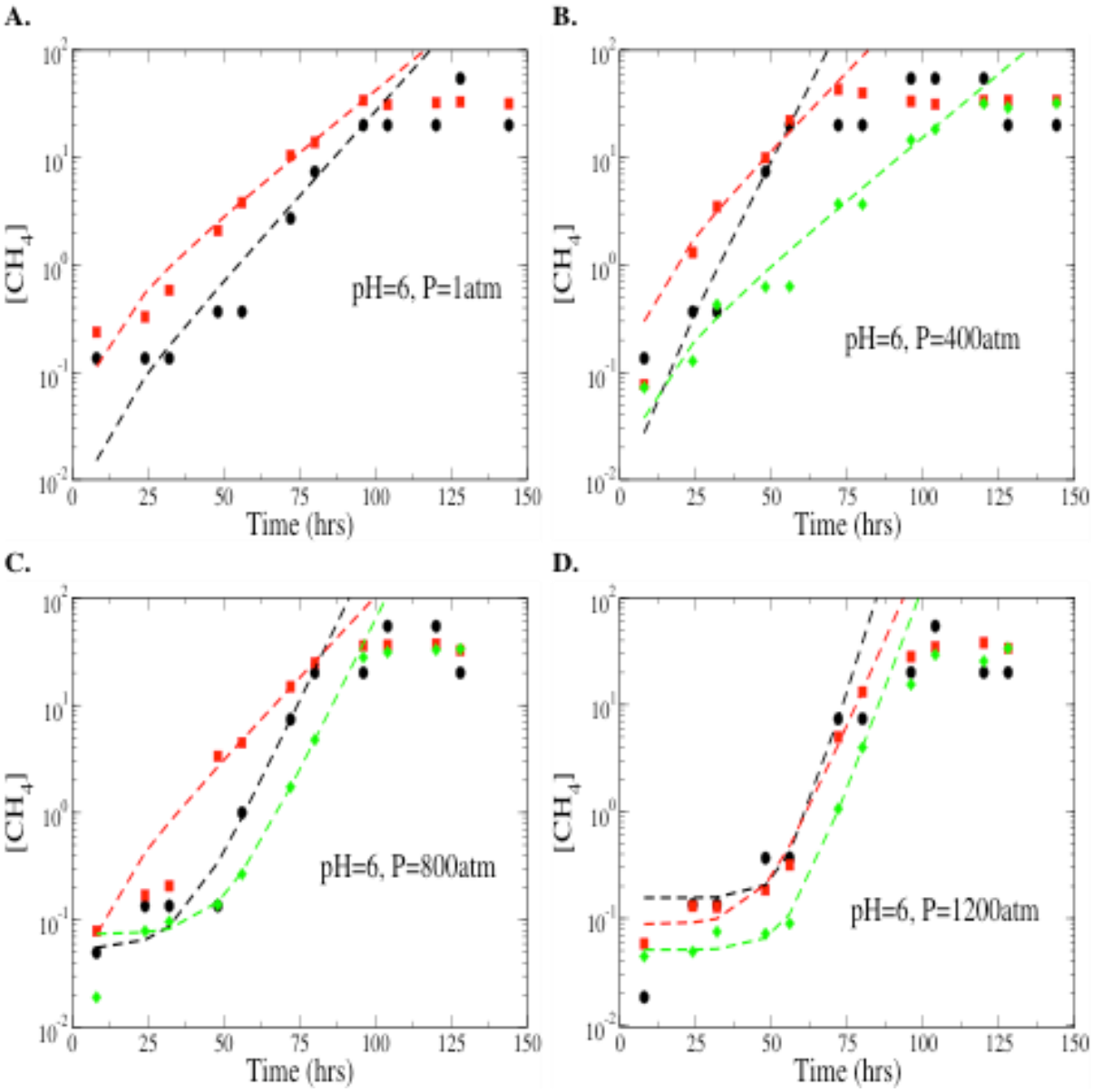

Figure 6.3: Growth curves represented by methane concentration produced by Methanothermobacter wolfeii as a function of time; Linear-log plot of methane production measured after exposure to pressures (A) P=1 atm (B) P=400 atm (C) P=800 atm (D) P=1200 atm, at pH=6 and temperature 55ºC. Symbols are the experimental data and color lines represent the fit using Eq. 1 through the data points in the exponential regime. Duplicates/triplicates are shown for all the pressures.

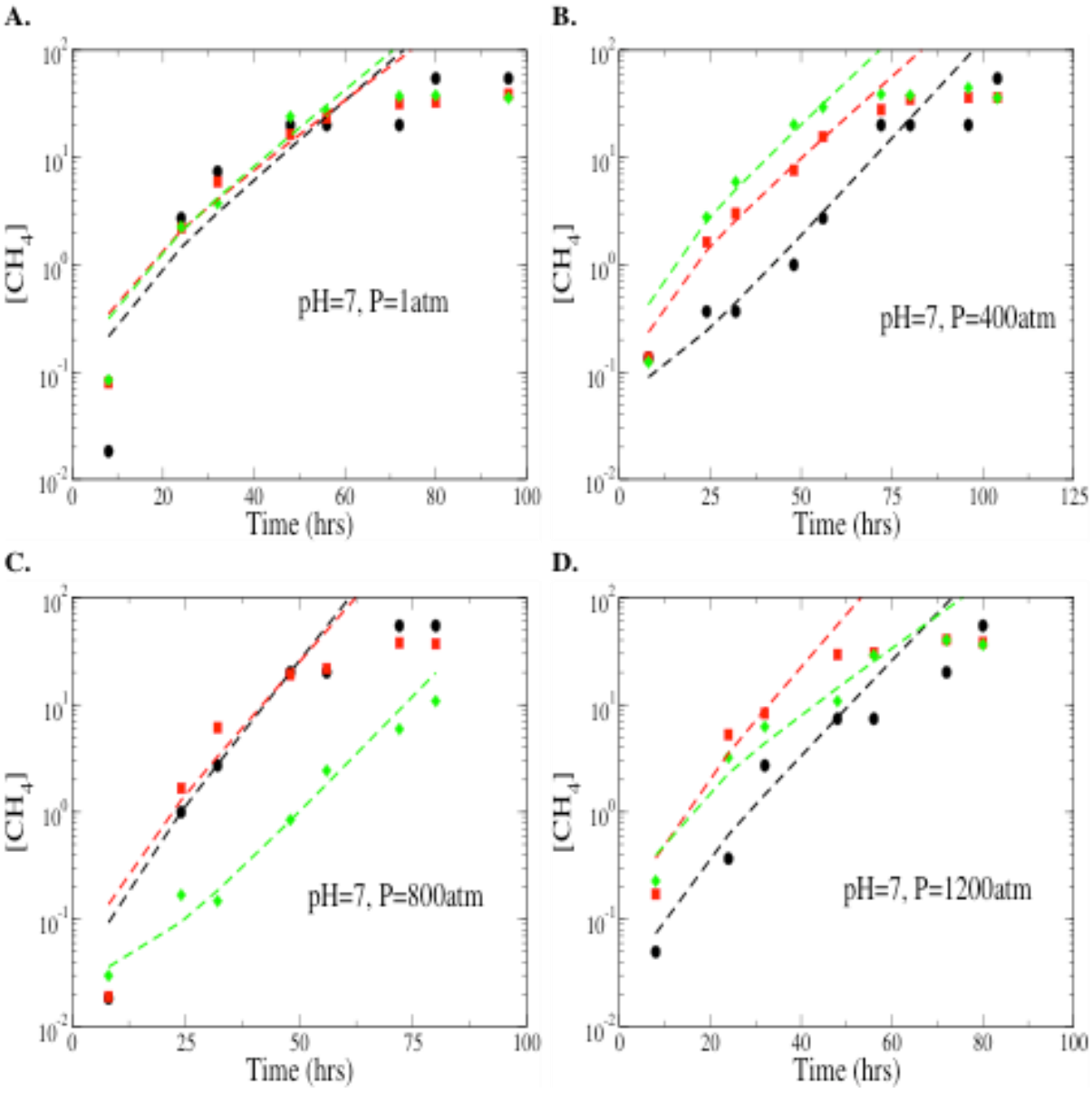

Figure 6.4: Growth curves represented by methane concentration produced by *Methanothermobacter wolfeii* as a function of time; Linear-log plot of methane production measured after exposure to pressures (A) P=1 atm (B) P=400 atm (C) P=800 atm (D) P=1200 atm, at pH=7 and temperature 55ºC. Symbols are the experimental data and color lines represent the fit using Eq. 1 through the data points in the exponential regime. Duplicates/triplicates are shown for all the pressures.

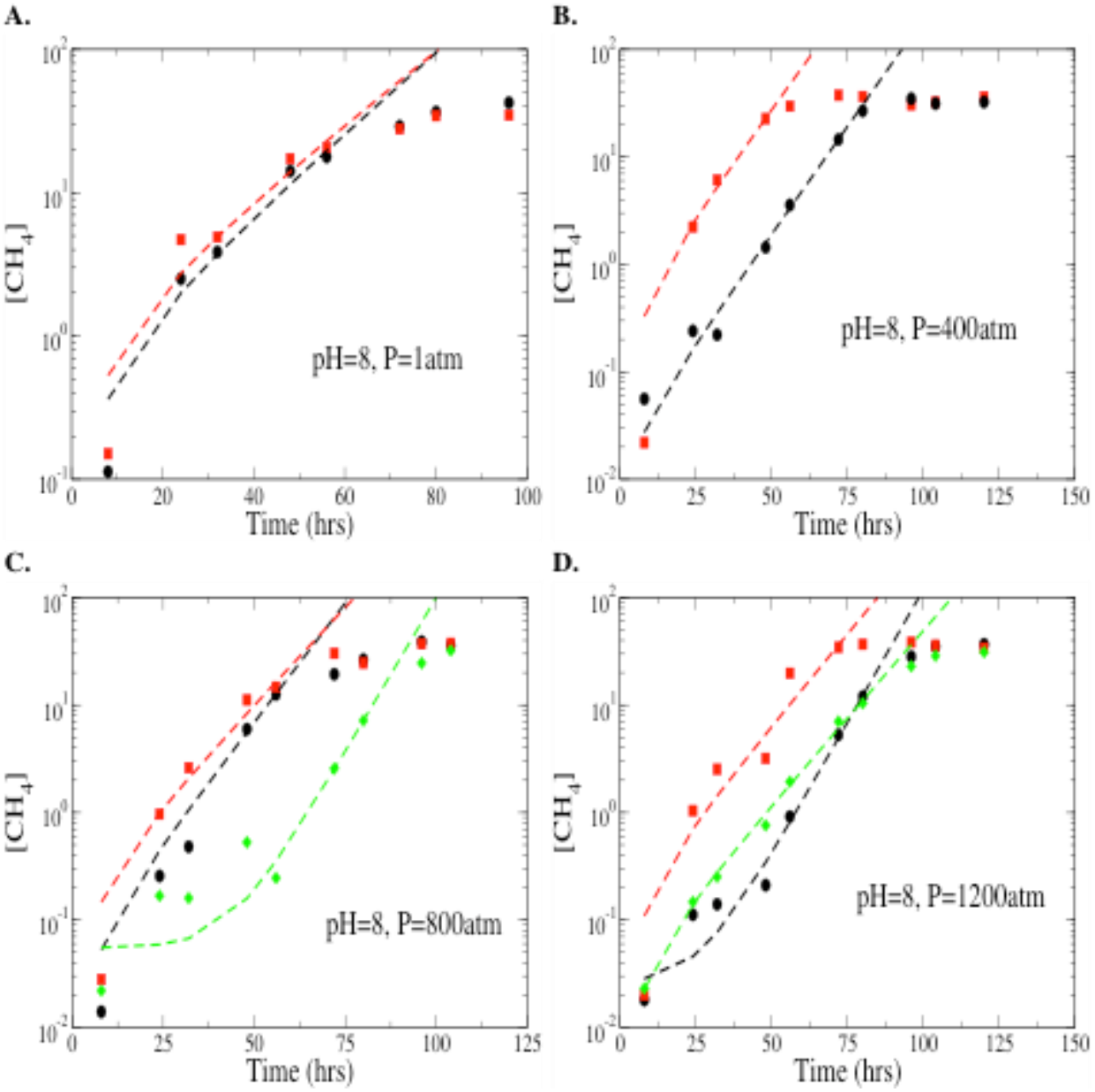

Figure 6.5: Growth curves represented by methane concentration produced by *Methanothermobacter wolfeii* as a function of time; Linear-log plot of methane production measured after exposure to pressures (A) P=1 atm (B) P=400 atm (C) P=800 atm (D) P=1200 atm, at pH=8 and temperature 55°C. Symbols are the experimental data and color lines represent the fit using Eq. 1 through the data points in the exponential regime. Duplicates/triplicates are shown for all the pressures.

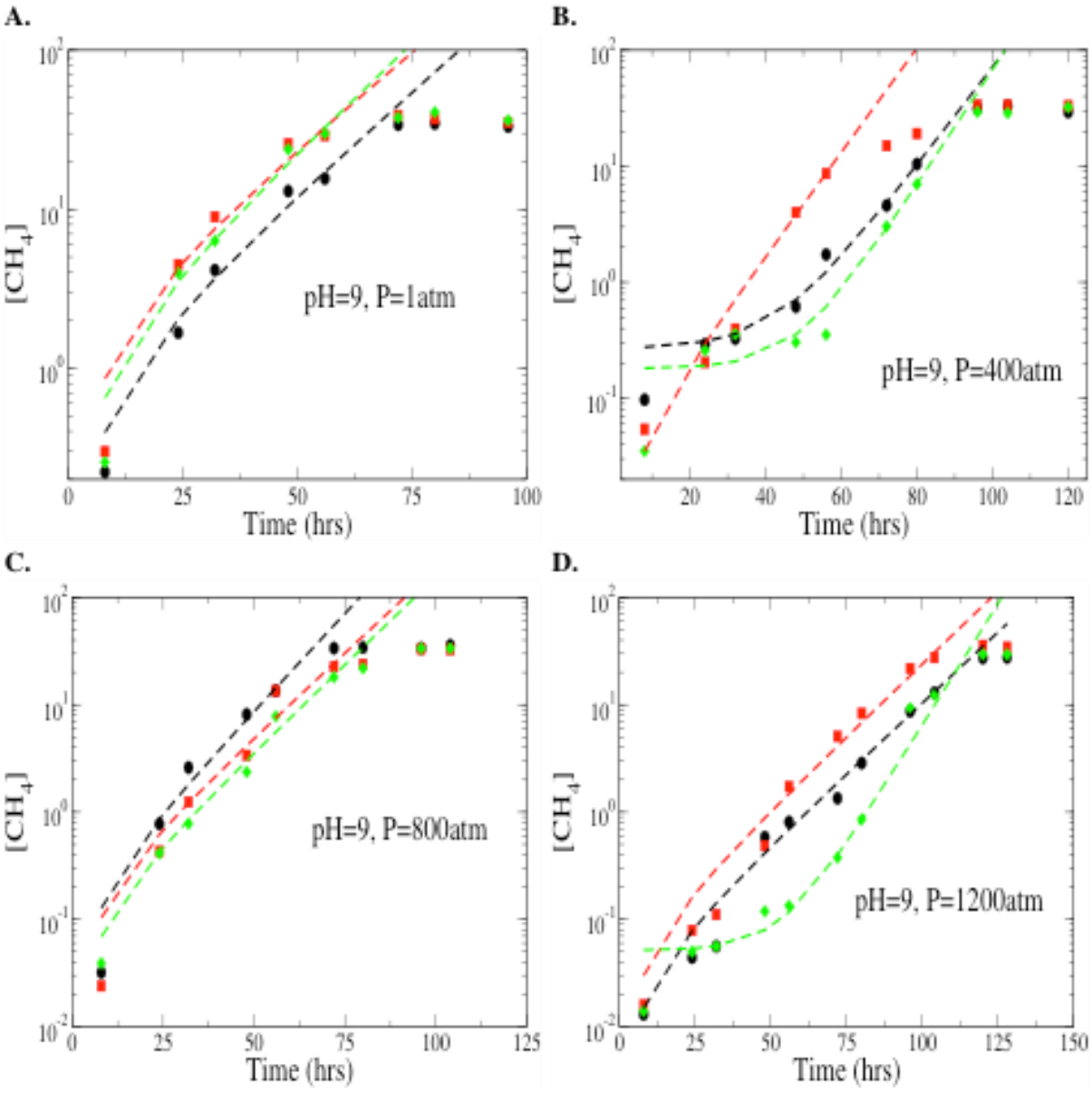

Figure 6.6: Growth curves represented by methane concentration produced by *Methanothermobacter wolfeii* as a function of time; Linear-log plot of methane production measured after exposure to pressures (A) P=1 atm (B) P=400 atm (C) P=800 atm (D) P=1200 atm, at pH=9 and temperature 55ºC. Symbols are the experimental data and color lines represent the fit using Eq. 1 through the data points in the exponential regime. Duplicates/triplicates are shown for all the pressures.

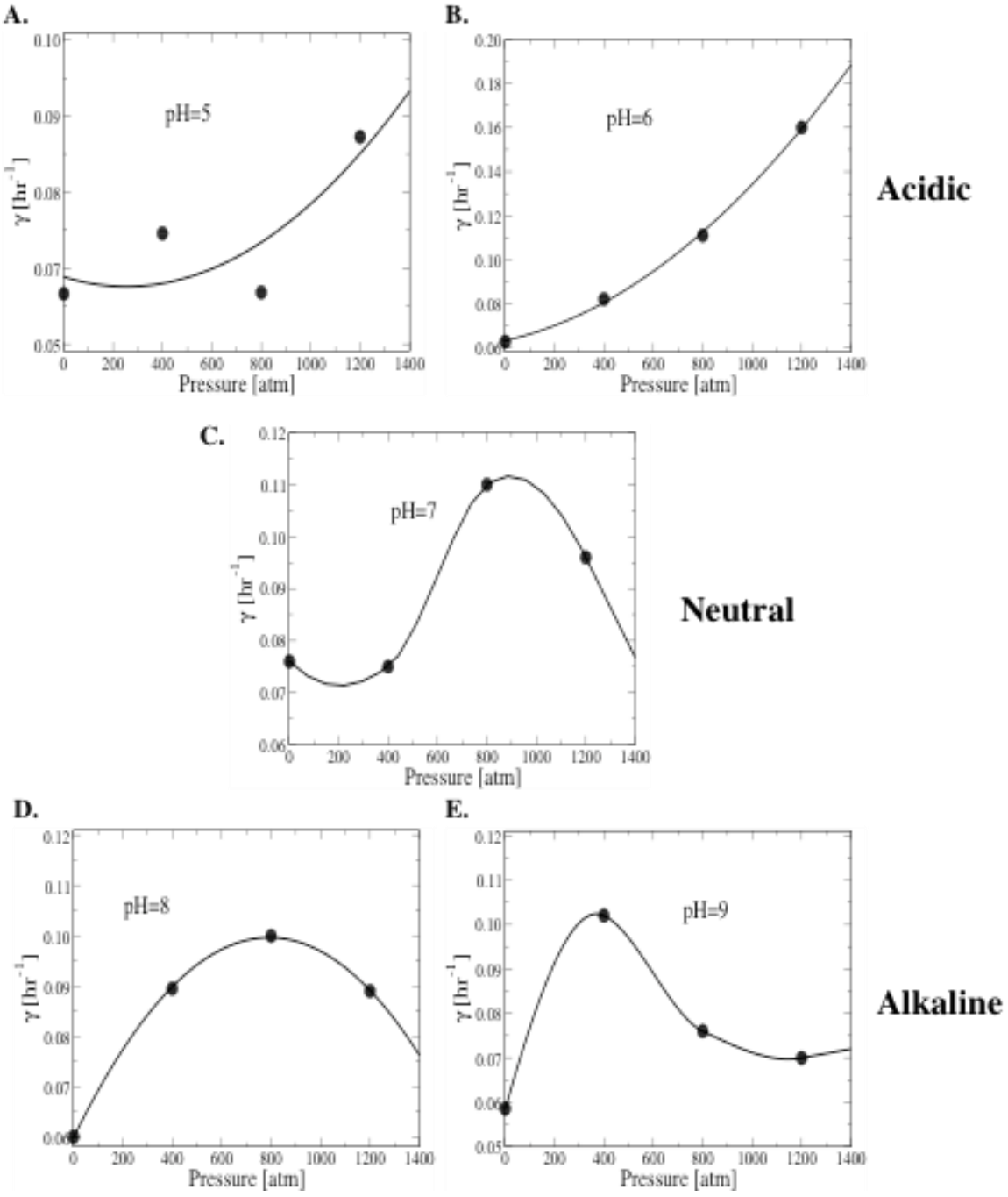

Figure 6.7: Growth rate, γ, of Methanothermobacter wolfeii as a function of exposure pressures. Growth rates were extracted from Figure 1 through 5. Each value is the average of duplicate/triplicate measurements.

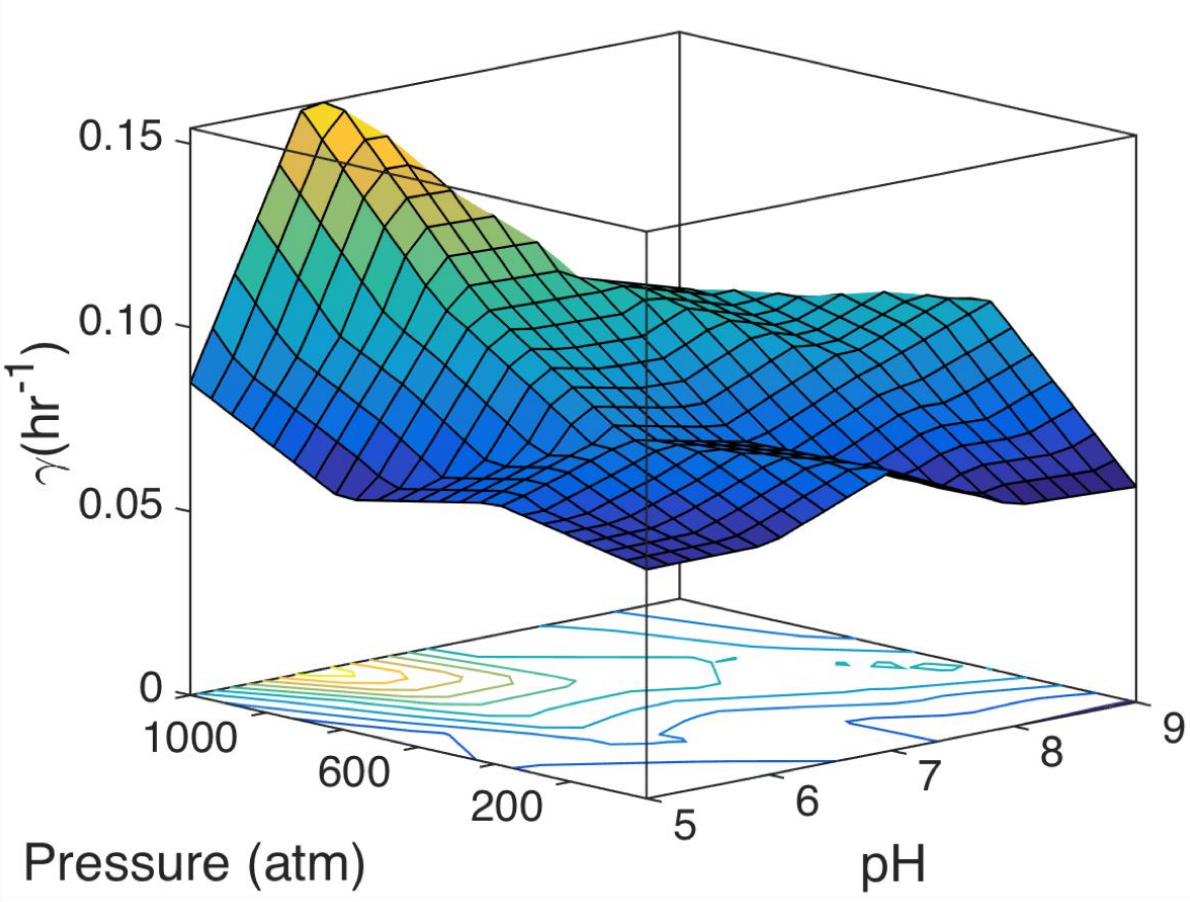

Figure 6. 8: Surface plot of γ as a function of pressure and pH.

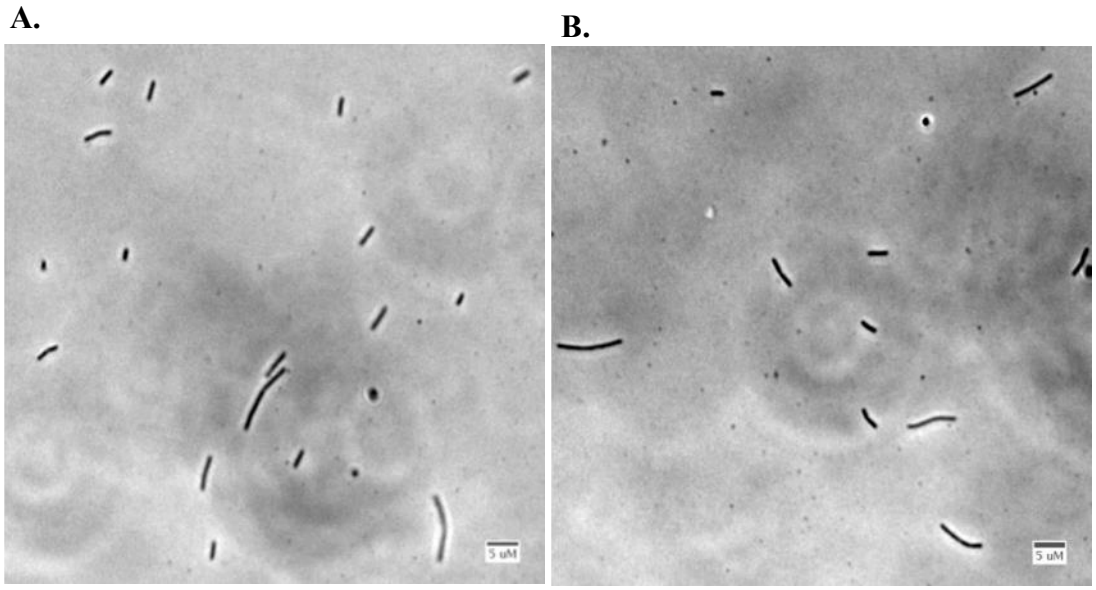

Figure 6.9: Morphology of Methanothermobacter wolfeii at (A) 1 atm and at (B) 1200 atm. Exposure to high pressure does not lead to a significant change in morphology of the cells.

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Chapter 7

Conclusions, Limitations, and Future Perspectives

7.1 Conclusions

This research has investigated the carbon isotopic biosignatures of metabolic methane produced by methanogens in simulated Martian geochemical environments, tolerance capability of the hydrated and desiccated cultures of methanogens to UVC radiation, and the growth and survivability of methanogens in presumed Martian deep subsurface conditions with respect to pH, temperature, and pressure. This work also strengthens the information that methanogens are not only the most promising candidates for possible life forms on/near the surface of Mars but also in the deep subsurface of this planetary body.

If life exists on the surface of Mars, it would have to respond to challenges of environmental extremes, such as low surface temperature, low atmospheric pressure, and DNA-damaging UV radiation. Therefore, the most feasible environments for active known life forms on Mars would be “near or deep subsurface”, where the temperature and pressure will be higher compared to the surface and the environment will be protected from the damaging cosmic radiations. Analogous to terrestrial subsurface microbial life, any Martian subsurface life has to contend with a wide range of physiological factors such as pH, pressure, and temperature.

The evidence such as the detection of sporadic methane in the Martian atmosphere, evidence of transient flow of liquid water, minerals, and elements on the Martian surface have supported the possibility of contemporary or ancient life on Mars. The sources of methane on Mars are still unknown. Stable carbon isotope fractionation is one of the several potential techniques to differentiate between biogenic and abiogenic sources of methane.

The second chapter describes the first goal for the identification and characterization of the stable carbon isotope fractionation of methane by methanogens growing on different Mars regolith analogs. Stable carbon ($^{13}C/^{12}C$) isotope fractionation values of metabolically produced methane by three different strains of methanogens such as Methanothermobacter wolfeii, Methanosarcina barkeri, and Methanobacterium formicicum were analyzed following growth in their respective growth-supporting media and on four different Mars regolith analogs, for example — JSC Mars-1, Artificial Mars Simulant, montmorillonite, Mojave Mars Simulant. M. wolfeii and M. barkeri have shown relatively consistent enriched values of ^{12}C on montmorillonite, a kind of clay found on Mars, compared to other Mars regolith analogs while M. formicicum showed subtle or negligible difference in carbon isotope fractionation values when grown on Mars analogs compared to the carbon isotope signature of methane in their respective growth media. This novel research could provide a piece of information towards the big enigma of sources of methane on Mars.

Chapter 3 discusses the second goal to determine the sensitivity of desiccated and hydrated cultures of halophilic Methanococcus maripaludis and non-halophilic methanogenic archaea, Methanothermobacter formicicum to DNA damaging UVC (254 nm) radiation for various time intervals. Hydrated M. maripaludis survived for 24 hours of UVC exposure, while in a desiccated condition, they endured for 16 hours. M. formicicum also survived UVC radiation for 24 hours in the liquid state, however, in a desiccated condition, the survivability of M. formicicum was decreased to 12 hours. Some shielding elements and minerals that could have protected cells from UVC radiations for limited time are discussed in this chapter. The results of this work imply that limited time exposure (12 – 24 hours) to UVC radiation on the

surface of Mars is not necessarily a limiting factor for the survivability of *M. maripaludis* and *M. formicicum*.

Chapters 4, 5, and 6 cover the third goal to examine the effects of several physical and chemical factors such as temperature, pressure, pH, and hydrogen concentration on the growth, survivability, and morphology of thermophilic methanogens, *Methanothermobacter wolfeii*. Interestingly, M. wolfeii had shown survivability to all temperatures (45º, 55º, 65º, and 75ºC), pressures (1, 400, 800, and 1200 atm), and pH (5, 6, 7, 8, and 9) and also in limited hydrogen concentrations. The stable carbon isotopic fractionation of biogenic methane produced by M. wolfeii, $\delta^{13}C(CH_4)$, at different temperatures and pressures was also analyzed and it was found that the carbon isotopic data showed slightly more enrichment in ^{12}C at conventional growth conditions—1 atm and 55ºC—than the carbon isotopic data obtained in non-conventional temperature and pressure. A comparison of the images of the cells before and after the exposure to various temperatures and pressures did not show any obvious alteration in the morphology of M. wolfeii. The results acquired in this research suggest that methanogens could be considered as good model candidates of life in near and deep subsurface Mars, especially *Methanothermobacter wolfeii*.

In conclusion, a few strains of methanogenic archaea were able to survive in partial simulated surface and the near/deep subsurface of Mars. The results obtained in this work aid our understanding in interpreting the carbon isotopic biosignatures and survivability of methanogens in various physicochemical surface and subsurface conditions of Mars. Furthermore, the outcome of this work could be used to assess real time measurements of methane as biosignatures due to metabolic activity of methanogenic archaea under simulated surface and subsurface environments of Mars.

7.2 Limitations and Future Perspectives

In this study, the experiments were performed under one or two Martian surface and subsurface conditions. It was difficult to mimic concrete surface or subsurface scenario of Mars in a laboratory setting. However, in order to examine growth and survivability of methanogens in actual conditions of Mars, it is important to consider one or two parameters at a time.

Also, in most experiments, metabolic methane concentrations produced by methanogens in the headspace gaseous samples were measured following exposure to various temperatures, pressures and pHs. The real time measurements of methane concentrations during methanogenesis were difficult due to anaerobic habitat of methanogens and technical limitations. In the future, with the advancement of technology, experiments could be performed to examine the effects of combined physical and chemical factors on the growth of methanogenic archaea in a Mars simulation chamber with surface and subsurface conditions. Appropriate cultures (hydrated or desiccated) of methanogens could be exposed in a Mars simulation chamber where appropriate Martian temperature, pressure, minerals, atmospheric gas composition could be maintained and the growth and survivability of methanogens can be investigated by measuring methane concentration simultaneously. Future advanced techniques could also help to calculate stable carbon isotope and hydrogen isotope fractionations of methane in real time.

Another limitation was the understanding of the survivability of methanogens on Mars soil simulants, under UVC radiation, non-optimal temperatures, pressures, and pH. In order to understand the survivability of methanogens under various physical and chemical conditions, it is important to study methanogens at their molecular or genomic levels. In other work, not reported in this study, one strain of methanogens did not show growth on some Mars soils simulants. The reasons that deter the growth of methanogens on Mars analogs are not known.

Furthermore, counting the number of cells in a liquid culture of anaerobic hydrogenotrophic methanogens was challenging. Therefore, it wasn't possible to determine the number cells that survived due to exposure to UVC radiation, and various temperatures and pressures. Methanogens used in this study could not be grown on solid media.

Future work can be done to characterize carbon and hydrogen isotopic composition of methane due to methanogenesis by varying parameters such as substrate concentration (changing H_2 and CO_2 concentration), temperature, and pressure along with the use of Mars soil simulants. The results obtained in this work can be used to compare the carbon and hydrogen isotopic data of methane obtained from the current or future missions of Mars.

Additionally, the search for a biosignature on different celestial bodies is one of the interesting and hot topics for finding extraterrestrial life. If methanogens have existed in the past or exist today either on niches of surface or subsurface Mars, there is a possibility of detecting biosignatures in the atmosphere and the planetary surface. In order to examine methanogenic biosignatures, future experiments can be performed by growing methanogenic archaea on different types of Mars regolith analogs in a Mars simulation chamber and studying the interaction of minerals with methanogens by using different techniques such as IR spectroscopy, Scanning Electron microscopy, and X-ray diffraction techniques. Methanogens could leave biosignatures in Mars regolith analogs and could alter the texture, composition and/or isotopic composition of the minerals or could form new minerals with methanogenic metabolic byproduct.