GROWTH AND SURVIVABILITY OF METHANOGENS AT HIGH PRESSURE AND HIGH TEMPERATURE: IMPLICATIONS FOR SUBSURFACE LIFE ON MARS Navita Sinha[1], Sudip Nepal[2], T. A. Kral[1,3], Pradeep Kumar[1,2] [1]Arkansas Center for Space and Planetary Sciences, University of Arkansas, Fayetteville, Arkansas, 72701, USA, [2]Department of Physics, University of Arkansas, Fayetteville AR 72701, [3]Dept. of Biological Sciences, SCEN 632, University of Arkansas, Fayetteville, Arkansas, 72701, USA. [nxs017l@uark.edu]

Introduction: Mars is an inhospitable planet because of the low atmospheric surface pressure (~7 mbar), low surface temperature (average of ~210K), intense DNA damaging radiation fluxes (1), presence of oxidizing compounds in the top soil (2), and other hostile conditions. Therefore, life, as we know it, on its surface is highly unlikely. However, the subsurface environments, which have relatively high pressure, high temperature, and protected from cosmic radiation, could provide hospitable settings for life. A number of evidences suggest that Mars was warmer and wetter a few billion years ago (3, 4). Also, it has been hypothesized that subterranean oases may exist today on Mars (5) and could provide a suitable environment for a subsurface biosphere. Additionally, the discovery and the study of subsurface extremophiles on Earth generated enthusiasm to find extinct or extant microbial life in the subsurface of Mars.

Methanogens are anaerobic archaea, which consume CO_2 and H_2 and produce CH_4 as their metabolic byproduct, and have been considered plausible Martian life-forms for a long time, even before the discovery of methane in the Martian atmosphere (6-10).

In this work, we examine growth and survivability of a methanogenic archaea, *Methanothermobacter wolfeii* , which grows optimally at about 55ºC, at four different pressures-- 1atm, 400atm, 800atm, and 1200atm and threee different temperatures-- 45ºC, 55ºC, and 65ºC. We have also characterized carbon isotope fractionation of headspace methane in order to investigate the effect of non-optimal temperatures and high pressures on the carbon isotope signatures.

Methods: A small volume (1ml) of fresh liquid culture of *M. wolfeii* (prepared in MM media) was transferred into a quartz cuvette (Spectracell) in an anaerobic chamber (Coy). The cuvette was then placed into a high hydrostatic pressure and temperature chamber (ISS, Illinois) 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 (11,12). A circulating water bath connected to high pressure-temperature chamber was used to control the temperature of the chamber. For a given pressure and temperature, the sample was kept in the high pressure-temperature chamber for 15 hours. Our experimental setup did not allow for pressurized hydrogen in the media unlike the normal growth protocol and only the dissolved hydrogen was present in the media for high pressure experiments.

After exposure to high pressure and temperature, 500uL of cells from the cuvette was then transferred into a vial containing 500uL of MM media. From this tube, 300 uL of sample was inoculated into three anaerobic tubes containing 10 mL of sterilized MM media. These anaerobic tubes were then pressurized with hydrogen gas and incubated at their optimum growth temperature at 55ºC and 1 atm pressure. A gas chromatograph (Shimadzu) was used to measure the methane concentration of the headspace gas of each of the samples periodically to assess the growth of *M. wolfeii* after the exposure to different pressure-temperature conditions. Optical density before and after loading the sample into high pressure-temperature chamber was also measured using a spectrophotometer. Phase contrast images of the cells were acquired using a Nikon microscope with 40x objective. The stable carbon isotope fractionation of methane in the headspace gas of all samples was measured by a Piccaro Cavity Ringdown Spectrometer G2201-i isotopic CO_2/CH_4 in the University of Arkansas Isotope Lab.

Results: A total of twelve experiments spanning a wide range of pressures and temperatures were performed. For all the pressures and temperatures studied here, *M. wofeii* exhibited methanogenesis when brought back to normal pressures. To assess methanogenic cells viability due to exposure to high pressures, we measured optical density before and after loading the sample into the high pressure-temperature chamber. In each experiment, we observed a slight decrease in optical density after the exposure to high pressures. To investigate if the exposure to high pressure changes the growth kinetics of cells, we measured methane concentration of *M. wolfeii* regrown at atmospheric pressure. In Figure 1(a), we show methane concentration as a function of time for the cells exposed to high pressures at temperature T=55ºC. We found that the saturation time for methane concentration varies with exposure pressure. Moreover, assuming an exponential growth of the cells, we extract the growth rate of cells exposed to all different pressures. In Figure 1(b), we show the growth rate (γ) extracted from (a) for all the pressures at T=55ºC. While $\boldsymbol{\gamma}$ does not change significantly until 400 atm, $\boldsymbol{\gamma}$ increases at higher pressures.

To assesss whether the growth conditions in the presence and in the absence of pressurized hydrogen in the media were different, we carried out control experiments of growth of *M. wolfeii* with and without

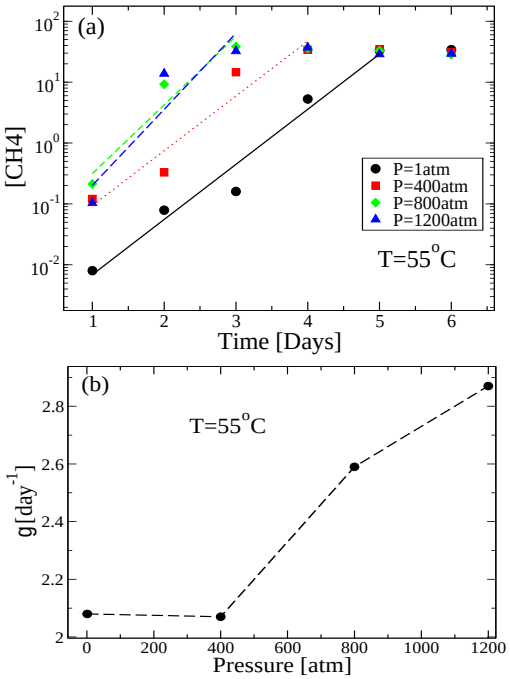

Figure 1. (a) Methane concentration of cells exposed to different pressures as a function of time at T=55ºC. (b) Growth rate of exposed cells as a function of exposed pressure obtained from (a).

pressurized hydrogen. In Figure 2, we show the optical density as a funtion of time in both conditions. We did not find any difference in growth between the absence and presence of pressurized hydrogen over the first 15 hours. However, we find that the growth at longer times varies and is dependent on the temperature. For T=45ºC, and 55ºC, we found that the growth is smaller in the absence of pressurized hydrogen. On the contrary, there is no difference in growth at T=65ºC.

Images of exposed cells were also analyzed to see morphological changes due to the effect of high pressures. We observed the increased number of elongated cells at higher pressures suggesting a lack of cell division.

Next, we have measured carbon isotope fractionation of headspace gaseous methane. We did not find any substantial change in their carbon isotopic values due to exposure of high pressures and different non-optimal temperatures. In Table 1, we have listed the carbon isotope fractation values on the fourth day after inoculations of exposed cells into the fresh MM media.

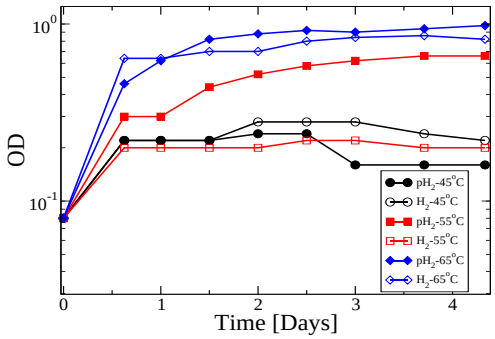

Figure 2. Optical density (OD) as a function of time at T=45ºC, 55ºC, and 65ºC and P=1atm with and without pressurized hydrogen in the media.

Pressure	*45ºC*	*55ºC*	*65ºC*
1atm	-69.12	-73.04	-69.77
400 atm	-72.54	-71.20	-69.69
800 atm	-72.51	-71.37	-69.38
1200 atm	-71.87	-70.14	-68.56

Table 1: Stable carbon isotope fractionation values (in per mil, ‰.) of methane produced by *M. wolfeii* cells exposed to different pressures and temperatures.

Discussion and Conclusions: We have investigated the survivability and growth of *M. wolfeii* in a wide range of pressures (1-1200 atm) and temperatures (45-65ºC) and interestingly, these thermophilic arachaea was able to survive at all the pressures and temperatures studied here. In the light of recent evidence of water and methane on Mars, our results suggest that methanogens could be a plausible life form that can survive and thrive at subsurface conditions.

Optical density measurement to quantify viability of cells is not reliable. Therefore, our future work will examine viability of cells (such as death rate) using a DNA intercalating dye DAPI. In order to create a pressure-temperature phase diagram of viability of methanogens, we will extend the range of temperature and pressure.

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