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Characterization of growth and photosynthesis of *Synechocystis* sp. PCC 6803 cultures under reduced atmospheric pressures and enhanced CO_2 levels

Eira Kanervo[1], Kirsi Lehto[1], Kurt Ståhle[1], Harry Lehto[2,3] and Pirkko Mäenpää[1]

[1]*Laboratory of Plant Physiology and Molecular Biology, Department of Biology, University of Turku, FIN-20014 Turku, Finland*
e-mail: kirsi.lehto@utu.fi
[2]*Tuorla Observatory, University of Turku, Väisäläntie 20, FIN-21500 Piikkiö, Finland*
[3]*Department of Physics, University of Turku, FIN-20014 Turku, Finland*

Abstract: Efficient life support systems are needed to maintain adequate oxygen, water and food for humans in extraterrestrial conditions. On the near-Earth missions, these are supplied by transport from the Earth, and by physical and chemical cleaning and recycling, but on long-term missions to far-away destinations, such as Mars, on-site production of the consumables may be required. Molecular oxygen and organic biomass can be most efficiently produced biologically, i.e. by photosynthesis. The conditions on Mars are distinctly harsh, and they strictly limit the growth and survival of any photosynthetic organisms to artificially maintained containments. For obtaining most economical growth conditions, minimal parameters need to be determined which still allow efficient growth of photosynthetic organisms. In this work we are testing how reduced air pressures (hypobaria) and increased CO_2 concentrations, i.e. features typical for Martian conditions, affect the durability, growth and photosynthesis of laboratory strains of cyanobacteria, a group of prokaryotic organisms capable of plant-like photosynthesis. Our preliminary results show that air pressures down to 0.1 atm or CO_2 concentrations up to 20% have no harmful effect on the photosynthetic oxygen production or growth rate of the cyanobacterial model species, *Synechocystis* sp. PCC 6803.

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Introduction

Within planetary and astrobiological research, manned missions to Mars or stations on that planet are foreseen. Under the Martian conditions, efficient environmental control and life support systems (ECLSS) are required to maintain suitable living conditions for humans, i.e. conditions similar to the Earth's surface in terms of air pressure, gas composition, temperature and protection against harmful radiation (Table 1). Also an adequate supply of food and water is required. So far, the supplies for all consumables in near-Earth space flights have been provided by transporting them from the Earth, and by maintaining them via different physical and chemical cleaning and recycling methods. However, such transport and on-site re-cycling may be inadequate, or too expensive for maintaining the required gas, food and water supplies in long-term missions to Mars. The only sustainable way would be to produce them on-site from the local resources. On Earth, photosynthesis by green plants, algae and photosynthetic bacteria produces molecular oxygen and organic biomass from atmospheric CO_2 and liquid water, with the energy being provided by sunlight.

Photosynthesis could also be utilized on the Martian surface, as CO_2 is available in the Martian atmosphere in excess, and water is available at least from the polar ice caps, and possibly also extractable from the subsurface water reservoirs or minerals. However, on Mars photosynthetic organisms need to be grown in specific enclosed conditions, to protect them from the harsh environment (Table 1), and also to prevent potential contamination of the Martian surface by any terrestrial organisms. In particular, higher plants are known to be very sensitive to high CO_2 concentrations, levels above 0.2% being harmful (Thayer 2005), and to low pressures, 0.1 atm or less being harmful (Paul *et al*. 2004), as well as to high levels of radiation. Thus, higher plant growth facilities on Mars would need to be maintained at, or near Earth-like conditions, with an air pressure and O_2 concentration much higher than those of the surrounding air. This would be technically very demanding, and therefore, it would be more feasible to utilize some more tolerant photosynthetic organisms, such as cyanobacteria, at

Table 1. *Average surface conditions on Mars and Earth (adapted from Graham (2004))*

Parameter	Mars	Earth
Surface gravity	0.38 g	1.00 g
Mean surface temperature	−60 °C	+15 °C
Surface temperature range	−145 to 20 °C	−60 to 50 °C
Insulation (PAR)	860 μmol of quanta/(m^2 s)	2000 μmol of quanta/(m^2 s)
UV radiation	>190 nm	>300 nm
Atmospheric pressure	5–10 mbar	1013 mbar
Atmospheric composition		
N_2	0.189 mbar, 2.7 %	780 mbar, 78 %
O_2	0.009 mbar, 0.13 %	210 mbar, 21 %
CO_2	6.67 mbar, 95.3 %	0.38 mbar, 0.038 %
Ar	0.112 mbar, 1.6 %	10.13 mbar, 1 %

Fig. 1. Underpressurized culture vials, used for *Synechocystis* cell cultivations, were located in a growth chamber. The CO_2-enriched, water-saturated air was led down into the bottles through plastic tubes. The liquid cultures were gently stirred (120 rpm) using magnetic stirrers under the bottles. A gauge controlled the vacuum.

least for the bulk production of molecular oxygen and organic biomass.

Cyanobacteria represent the largest, most diverse and ecologically most successful group of micro-organisms. This group of prokaryotes occupies a wide range of illuminated habitats in terrestrial, freshwater and marine environments (Rippka 1988). Many cyanobacterial species are adapted to growth in the most extreme conditions on Earth, including cold waters (Richardson *et al*. 2000; De los Rios *et al*. 2003; Taton *et al*. 2003; Cockell & Stokes 2004), hot deserts and hot springs (Stetter 1999) and hyper-saline and hyper-alkaline environments (Rippka 1988; Oren 2002; Pogoryelov *et al*. 2003). They also thrive under the high ultraviolet (UV) radiation conditions of the Arctic and Antarctic (Graham 2004). It was recently reported that some cyanobacteria can also tolerate high concentrations (up to 100 %) of CO_2, at least for short periods of time (Thomas *et al*. 2005). The ancestors of cyanobacteria are considered to be the producers of the first bulk amounts of oxygen in the developing terrestrial atmosphere (Blankenship 1992). Consequently, they also contributed to the formation of the ozone layer, which protected the Earth from UV radiation and facilitated the development of terrestrial life forms. Structurally cyanobacteria represent several forms ranging from unicellular species to multicellular filamentous ones, some of them possessing specific cell types, e.g. for fixation of molecular nitrogen (Rippka 1988). Some cyanobacterial species are free-living, some are colonial, while other species exist in symbiotic associations with other micro-organisms, algae and higher plants.

Synechocystis sp. PCC 6803 (from here on referred to as *Synechocystis*) is a unicellular freshwater cyanobacterium that is one of the most frequently used cyanobacterial species in photosynthesis research. Due to its easy cultivation, amenability to genetic manipulation and its completely sequenced genome (Kaneko *et al*. 1996), *Synechocystis* has been used as a favourite model organism in studies of oxygenic photosynthesis and other metabolic processes (Pakrasi 1995), as well as in molecular biological responses, e.g. to low temperature (e.g. Somerville 1995; Kanervo *et al*. 1997). However, the effects of some experimental parameters, such as low atmospheric pressure, on the growth of *Synechocystis* have not been tested so far. Therefore, in this study we have tested the adaptability of the wild-type strain of *Synechocystis* to low atmospheric pressure and to high concentrations of CO_2.

Materials and methods

Cells of the wild-type *Synechocystis* sp. PCC 6803 were grown photoautotrophically under constant illumination at the photosynthetic photon flux density of 40 μmol (2.4×10^{19}) photon m^{-2} s^{-1}, at 32 °C, in the BG-11 growth medium (Stanier *et al*. 1971; Rippka *et al*. 1979), which was supplementally buffered with 20 mM Hepes-NaOH, pH 7.5. The cell suspensions were grown in 7-litre vacuum glass bottles and placed on magnetic stirrers, the whole system being located in a growth chamber (Fig. 1). In the experiments three bottles were used, in which three separate replicate cultivations could be grown at the same time under the same conditions. The air pressure was adjusted in these bottles by a vacuum pump. The concentrations of CO_2 were adjusted by a flow-meter from an external gas supply, from which the gas was delivered to the bottles via thin plastic tubes extending from the main tubes into the bottles, and leading the gas

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down to the cell suspensions. In the experiments, air pressures of 0.1 atm, or of 1 atm were used in combination with different concentrations of CO_2 or N_2. The duration of the experiments was either 3 or 9 days. The 9-day experiments were conducted either with brief opening of the bottles four times during the cultivation (to take samples for measurements of oxygen evolution) or without opening the bottles. After the 9-day experiments, the cultivations were grown further for 4 or 5 days under normal growth conditions (recovery conditions). In the 0.1 atm air pressure treatments, the gas was led into the bottles through water, to make it saturated with water and to reduce the evaporation of the culture liquid.

The growth of the cells was determined by measuring the absorbance of the cell suspension at 750-nm wavelength (A_{750}) with a spectrophotometer, a method routinely used to estimate chlorophyll *a* concentration and reflecting *in vivo* cell growth. From the A_{750} value the exact chlorophyll concentration can also be determined (Bennett & Bogorad 1973) if required. Photosynthesis was measured as *in vivo* oxygen evolution of photosystem II under saturating light with a Clark-type oxygen electrode (Hansatech). For each measurement, an aliquot of cell culture, containing 10 μg chlorophyll, was collected by centrifugation and resuspended in 1 ml of fresh BG-11 medium and placed in the measuring cuvette. As an electron acceptor, 0.25 mM 2,6-dichloro-*p*-benzoquinone was added to the cell suspension and 0.25 mM ferricyanide was added to keep the benzoquinone in an oxidized form. The photosynthetic activity of the cells was measured in this device as the amount of oxygen diffusing out of the cuvette, through a semi-permeable membrane. The photosynthetically produced O_2 becomes reduced into electrolytes on the platinum cathode, and thus mediates electric current, which is directly related to the amount of oxygen produced (see http://www.eutechinst.com/techtips/techtips16.htm).

Results and discussion

Synechocystis cells survived the 9-day incubation under the reduced air pressure of 0.1 atm, with no added CO_2. However, the cells did not grow under these conditions, while the cell density of the control cultivation, maintained at ambient air pressure, increased fivefold (Fig. 2).

As no growth was observed at an air pressure of 0.1 atm during the 9-day cultivation, we wanted to test whether the cells still maintained their capacity for photosynthetic oxygen evolution. For this purpose the vials were briefly opened at 3-day intervals, samples were withdrawn, and their light-saturated capacity for photosynthetic oxygen evolution *in vivo* was determined by the oxygen electrode. After 4 days of liquid cultivation, the value for photosynthetic oxygen evolution was typically 107 μmol of oxygen produced/mg chl/h, which was about equal to the photosynthetic activity of the control cells maintained under ambient conditions (90 μmol of oxygen produced/mg chl/h (standard deviations were within 10%). This indicated that the photosynthetic machinery remained completely functional under 0.1 atm

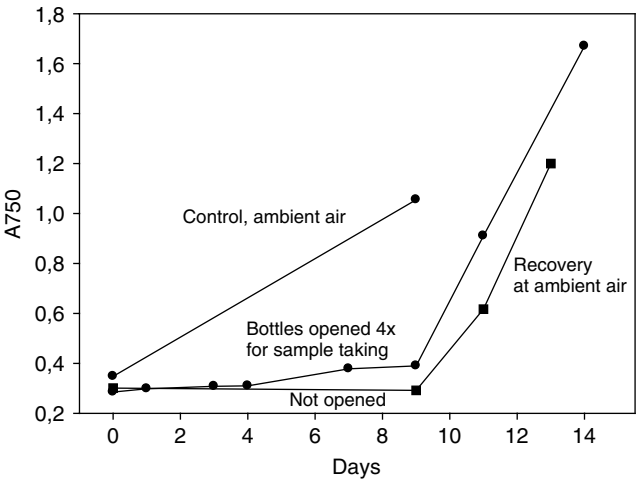

Fig. 2. *Synechocystis* growth curves of the 9-day cultivations, at 0.1 atm, without CO_2 enrichment, at 32 °C. Cell densities were measured spectrophotometrically as A_{750}. After the 9-day cultivation, the cell suspensions were transferred to normal growth conditions (ambient air), to detect recovery from the low-pressure stress (for further details see the text).

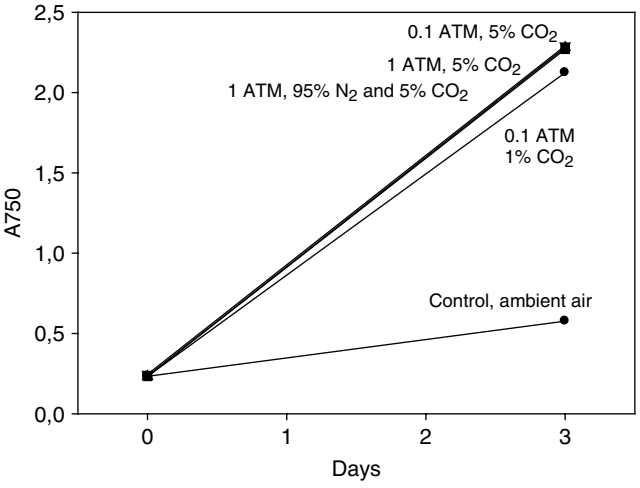

Fig. 3. *Synechocystis* growth curves for the 3-day cultivations at 0.1 or 1 atm with 1% or 5% CO_2 enrichment, or with 5% CO_2 and 95% N_2, at 32 °C. Cell densities were measured spectrophotometrically as A_{750} (for further details see the text).

pressure during the 9-day cultivation. The functionality of the cells under these conditions was also indicated by their very rapid growth when returned back to ambient air, to test their potential to recover from the low-pressure stress: upon transfer of the cells to recovery conditions, they directly entered explosive growth (Fig. 2). Also the transient releases of the vacuum treatment slightly increased the cell growth, as compared to the continuous low-pressure treatment, apparently due to the small increase in the available CO_2 (Fig. 2). These results indicate that the reduction of the cell growth at the 0.1 atm air pressure was solely due to the reduced availability of CO_2 under the experimental conditions studied.

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Increasing the CO_2 concentration up to 0.4% under the same low-pressure conditions of 0.1 atm (corresponding to a 0.04% partial pressure of CO_2 in ambient air), more than doubled the cell growth, as compared to the control culture (data not shown). When the CO_2 concentration of the air was further increased to a 1% or 5% level at 0.1 atm pressure, the cell growth was about four times higher than in the control culture, as measured by the A_{750} absorbance (Fig. 3). A similar growth increase was also produced by 5% CO_2 at 1 atm pressure in otherwise normal air, or by 5% CO_2 in the 1 atm pressured air with oxygen depleted and replaced by nitrogen (Fig. 3).

Our results allow us to conclude that *Synechocystis* tolerates the low atmospheric pressure of 0.1 atm, provided the CO_2 concentration is not limiting. Our preliminary results also indicate that *Synechocystis* is able to grow under CO_2 concentrations of up to 20%, or even higher (data not shown). Consistently, the high tolerance of *Synechocystis, Synechococcus, Anabaena* and *Plectonema boryanum* to CO_2 has also been shown recently (Thomas *et al.* 2005). Currently we are continuing our work focusing especially on CO_2 concentrations higher than 20% and on atmospheric pressures lower than 0.1 atm, which are more similar to Martian conditions.

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