Temperature-dependent growth rate and photosynthetic performance of Antarctic symbiotic alga *Trebouxia* sp. cultivated in a bioreactor

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**Abstract**

Optimum growth temperature of *Trebouxia* sp. (re-classified as *Asterochloris* sp. recently), a symbiotic lichenized alga was evaluated using a batch culture cultivated in a bioreactor. The algae were isolated from lichen thalli of *Usnea antarctica* collected at the James Ross Island, Antarctica in February 2012. The algae were isolated under laboratory conditions and then cultivated on agar medium at 5°C. When sufficiently developed, the algae were suspended in a BBM liquid medium and cultivated in a photobioreactor for 33 days at either 15, or 10°C. During cultivation, optical density (OD) characterizing culture growth, and effective quantum yield of photosystem II (Φ<sub>PSII</sub>) characterizing photosynthetic performance were measured simultaneously. Thanks to higher Φ<sub>PSII</sub> values, faster growth was achieved at 10°C than 15°C indicating that *Trebouxia* sp. might be ranked among psychrotolerant species. Such conclusion is supported also by a higher specific growth rate found during exponential phase of culture growth. The results are discussed and compared to available data on temperature-dependent growth of polar microalgae.

**Key words:** *Usnea antarctica*, chlorophyll fluorescence, lichen, effective quantum yield, James Ross Island, psychrotolerance, *Asterochloris*

**Abbreviations:** Φ<sub>PSII</sub> - effective quantum yield of photosystem II, μ - specific growth rate, OD - optical density, PBR<sub>r</sub> - photobioreactors

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Introduction

*Trebouxia* sp. is a green unicellular microalga. It belongs to division Chlorophyta, class Trebouxiophyceae, order Trebouxiales. Recently, it is re-classified as *Asterochloris* (Peksa & Škaloud 2011). Although there are various types of photobionts in the lichens, algae of genus *Trebouxia* sp. are well known as a photobionts presented in two thirds of all lichen species. It is found also in extremophilic lichens growing in Antarctica, including *Usnea antarctica*, *U. sphacelata* and *U. aurantiaco-atra*, most common fruticose species found at the islands on the west (South Shetlands, Argentine Islands) and east side of the Antarctic Peninsula (James Ross Island group). *Trebouxia* sp. is a typical photobiont for the lichens of genus *Usnea*, however, *Poterioochromonas* sp. is recently reported as rare co-occurring symbiont in *Usnea longissima* (He et Zhang 2012). In general, cells of *Trebouxia* sp. has about 10 – 15 μm in diameter. There is about 26 species in this genus.

*Trebouxia* sp. reproduces asexually by autospores or zoospores. Sexually reproductive stages have not been observed directly in any genus of class Trebouxiophyceae (Lewis et McCourt 2004). Species of genus *Trebouxia* differ in morphology of plastids and structure of pyrenoids (Friedl 1989). Many species of genus *Trebouxia* has been studied, but there is still little known about its physiological characteristics and photosynthetic parameters. However, there is a basic knowledge about its photosynthetic responses to heavy metal induced stress (e.g. Branquinho et al. 2011, Piovar et al. 2011), high light stress (Showman 1972), dehydration stress (Wieners et al. 2012), osmotic (Váczi et Barták 2006) and oxidative-induced stress (Del Hoyo et al. 2011). The alga is in many cases studied as a part of lichen thalli (e.g. Alvarez et al. 2012, Branquino et al. 2011).

*Trebouxia* sp. algae are considered either psychrophilic or psychrotolerant. Psychrophilic algae grow at temperatures below 0 °C and typically have temperature growth maxima only a few degrees above zero. Typical psychrophilic algae, such as e.g. genera *Chlamydomonas* and *Chloromonas* are found in polar regions. They have growth optimum below 10°C. In spite of the fact that lichenized *Trebouxia* thrives well in cold polar environments, its growth optimum is reported above 15°C (Kvíderová et al. 2010). Therefore, it may be ranked rather psychrotolerant than psychrophilic.

For long-term storage, symbiotic algae are mostly cultivated on agar plates in Petri dishes. Cultivation on agar plates is also used to develop cultures suitable for determination purposes (Gärtner & Inolić 1998). Cultures on agars and/or nitrocellulose disks are frequently used in the experiments studying stress-induced physiological responses, such as e.g *Trebouxia* sensitivity to heavy metals (Piovar et al. 2011), photosynthesis sensitivity to low temperature and drought stress (Sadowsky & Ott 2012). Another possibility is to cultivate the alga in a liquid medium. For cultivation of lichen symbiotic algae, a variety of liquid media and volumes of cell culture flasks is used. This approach was applied in the experiments studying dehydration (Wieners et al. 2012, Gasulla et al. 2013), and oxidative stress effects (Del Hoyo et al. 2011). Recently, photobioreactors (PBRs) are used in cultivation of numerous algal species. There are many types of photobioreactors – e.g. large open ponds, enclosed flat-plate PBRs, vertical column PBRs, panel PBRs and smaller indoor PBRs. Algae cultivated in large outdoor photobioreactors can be used for the production of some bioactive substances (Fu et al. 2012). Small indoor bioreactors are often used for laboratory experiments or growth analysis of algal
cultures using different indicators, such as e.g. changes in various physiological parameters caused by cultivation temperature and/or photosynthetic radiation. There are some advantages of using these bioreactors for algae cultivation such as e.g. the possibility of automatic continual measurements of physiological parameters during the whole cultivation period. In our study, we used a PBR for long-term cultivation in liquid medium to evaluate temperature optimum.

Material and Methods

*Trebouixia* sp. was isolated from an Antarctic lichen *Usnea antarctica* collected at the James Ross Island (63.81 S, 57.83 W). The isolation was done by a gradient centrifugation method according to Gasulla et al. (2010) using a Percoll®. After isolation, the alga was cultivated on agar medium at 5°C. When algal culture was sufficiently developed, it was collected from the surface of agar medium and suspended in a liquid medium (nitrogen-enriched anorganic Bold’s Basal Medium, 3N-BBM, Ahmadjian 1993). Then, it was cultivated in a FMT-150/400 photobioreactor (PSI, Czech Republic) for 33 days. For cultivation, two different growth temperatures (15°C, 10°C) and 16/8 h light/dark period were used (70 μmol m⁻² s⁻¹). The photobioreactors had a flat-vessel design that enabled uniform illumination within the whole volume of cultivated culture of *Trebouixia* sp. During cultivation, optical density (OD at 680 nm) of *Trebouixia* sp. culture, and effective quantum yield (ΦPSII) of photosynthetic processes in photosystem II were measured repeatedly. This was possible thanks to integrated densitometer that measured light scattering at 680 nm and a fluorometer that measured fluorometric signals Fm’ and Fs from which ΦPSII was calculated (ΦPSII = [Fm’- Fs] / Fm’, Genty et al. 1989). Finally, OD and ΦPSII were plotted against the time of cultivation, so that temperature-induced differences in growth rate and photosynthetic efficiency could be distinguished. The relations of OD and ΦPSII to the time of cultivation were fitted by S-curves using the below log-logistic model with five parameters:

\[
f(x) = C + \frac{D - C}{1 + \left(\frac{x}{E}\right)^B}F
\]

where B is Hill slope, C is minimum value, D is maximum value of dependent variable, E is C₅₀, and F is parameter describing asymmetry of S-curve.

Since the values of OD were measured repeatedly throughout cultivation time, specific growth rate (μ) was calculated for 50 h subperiods using the below equation:

\[
μ = (N₂ - N₁) / t₂-t₁
\]

where t₂, t₁ are the times within the exponential phase of culture growth (t₂>t₁, t₂-t₁= 50 h). N₂ is optical density (OD) derived from the fitted S curve at t₂, N₁ is OD at t₁.
Fig. 1. *Trebouxia* sp. cells isolated from *Usnea antarctica* collected at the James Ross Island, Antarctica. © Photo: K. Trnková.

| Estimate | Std.Error | t-value | p-value |
|----------|-----------|---------|---------|
| b:1      | -0.43     | -3.30   | 0.00    |
| c:1      | 0.13      | 19.60   | 0.00    |
| d:1      | 4.73      | 0.92    | 0.36    |
| e:1      | 554.45    |         |         |
| f:1      | 4.91      |         |         |

| Estimate | Std.Error | t-value | p-value |
|----------|-----------|---------|---------|
| b:2      | -46.91    | -1.90   | 0.06    |
| c:2      | 0.12      | 22.49   | 0.00    |
| d:2      | 0.41      | 105.44  | 0.00    |
| e:2      | 490.47    | 66.17   | 0.00    |
| f:2      | 0.06      | 1.83    | 0.07    |

Table 1. Generalized log-logistic parameters of S-curve (see Eqn. 1) fitting data points of optical density (OD) of *Trebouxia* sp. culture as dependent on the time of cultivation.

| Estimate | Std.Error | t-value | p-value |
|----------|-----------|---------|---------|
| b:1      | 15.85     | 0.96    | 0.34    |
| c:1      | 0.55      | 88.03   | 0.00    |
| d:1      | 0.61      | 74.38   | 0.00    |
| e:1      | 439.89    | 1.73    | 0.09    |
| f:1      | 21.87     | 0.13    | 0.90    |

| Estimate | Std.Error | t-value | p-value |
|----------|-----------|---------|---------|
| b:2      | -189.89   | -1.04   | 0.30    |
| c:2      | 0.68      | 97.31   | 0.00    |
| d:2      | 0.85      | 126.09  | 0.00    |
| e:2      | 429.84    | 99.69   | 0.00    |
| f:2      | 0.22      | 0.84    | 0.40    |

Table 2. Generalized log-logistic parameters of S-curve (see Eqn. 1) fitting data points of effective quantum yield of PS II ($\Phi_{PSII}$) of *Trebouxia* sp. culture as dependent on the time of cultivation.

Results

Time courses of OD showed that faster growth of *Trebouxia* sp. was achieved at 10°C than 15°C. At 10°C, the culture OD showed typical S-curve, with maximum growth rate found after 420 h of cultivation. At 15°C, however, the OD values exhibited rather constant growth rate (see Fig. 2, Table 3). Final culture density was higher when cultivated at 10°C (OD = 0.45) than 15°C (OD = 0.35) indicating that optimum cultivation temperature for *Trebouxia* sp. is definitely below 15°C. Such conclusion is supported also by fluorometric data. While effective quantum yield ($\Phi_{PSII}$) reached relatively lowered value ranging from 0.5 to 0.7 at
15°C, it was much higher at 10°C (0.6-0.8). Moreover, $\Phi_{\text{PSII}}$ showed increasing values with cultivation time indicating progressive acclimation to cultivation conditions (10°C), highly effective performance of photosynthetic apparatus at such more favourable temperature. At 15°C, contrastingly, $\Phi_{\text{PSII}}$ exhibited slightly decreasing trend with cultivation time. This finding supports the conclusion that 15°C is suboptimal cultivation temperature for Trebouxia sp. cultured in liquid medium.

| Days of cultivation | Cultivation temperature |
|---------------------|-------------------------|
|                     | 15°C        | 10°C        |
| **Intervals (h)**   | $\mu_{15}$ | $\mu_{10}$ |
| 0 - 50              | 0.0001     | below 0.0001|
| 50 - 100            | 0.0002     | below 0.0001|
| 100 - 150           | 0.0003     | 0.0001      |
| 150 - 200           | 0.0003     | 0.0003      |
| 200 - 250           | 0.0003     | 0.0004      |
| 250 - 300           | 0.0003     | 0.0006      |
| 300 - 350           | 0.0003     | 0.0008      |
| 350 - 400           | 0.0003     | 0.0010      |
| 400 - 450           | 0.0003     | 0.0013      |
| 450 - 500           | 0.0003     | 0.0012      |

Table 3. Specific growth rate ($\mu$) derived from optical density (OD) measurements of Trebouxia sp. cultured in a photobioreactor at 10 and 15°C.

**Discussion**

In this study, an optimum growth temperature of Trebouxia sp. cultivated in batch culture was studied using two cultivation temperatures (10 and 15°C). Faster growth found for Trebouxia sp. cultivated at 10°C is well comparable to the results of Teoh et al. (2004) who investigated growth of several Antarctic microalgae in response to temperature. The authors reported the highest specific growth rates for temperature ranging from 6°C to 14°C. Similarly, earlier study of Seaburg et al. (1981) showed that optimum growth temperature for 35 species of Antarctic microalgae ranged between 7.7 to 18°C. Fogliano et al. (2010) reported that Koliella antarctica, an unicellular extremophilic green alga from the Ross Sea, Antarctica, exhibited higher growth rates and biomass production in 15°C than in 10°C of cultivation temperature. This seems to be contradictory to our findings since in Trebouxia sp., there was faster growth and OD values found in 10°C than in 15°C (see Fig. 2). However, Fogliano et al. (2010) measured only the initial phase of growth (10 d), while our results showed completed S-curves reaching maximum constant value of OD because of long-term cultivation (more than one month). During the first 7 day of cultivation Trebouxia sp., similarly to K. antarctica exhibited higher
Fig. 2. Time courses of optical density (OD, the upper panel), and effective quantum yield of photosystem II ($\Phi_{\text{PSII}}$, the lower panel) in the culture *Trebouxia* sp. cultivated in a photobioreactor at two growth temperatures: 10°C (open symbols), and 15°C (full symbols).
OD in 15°C, while further cultivation led to more rapid growth in 10 then 15°C. Some unicellular algae from Antarctica, however, exhibit higher growth rates in cultivation temperature above 20°C, as reported by Chen et al. (2012) for Stichococcus (Tre bouxiophyceae) and Blanc et al. (2012) for polar alga Coccomyxa subellipsoidae.

In our experiment, Trebouxia sp. exhibited higher values of \( \Phi_{\text{PSII}} \) and resulting biomass at the temperature of 10°C throughout the cultivation. Therefore, the Trebouxia strain isolated from lichen thalli of Usnea antarctica could be ranked among psychrophilic algae because similar responses, i.e. optimum growth at temperature below 15°C is reported by Vona et al. (2004) for cryophilic alga Koliella antarctica. Such conclusion may be supported also by the study of Ocampo-Friedmann et al. (1988) who classified two species of genus Trebouxia from Antarctica as psychrophilic algae with temperature optimum around 17.5°C. The algae of genus Trebouxia, however, are present in many lichen species distributed worldwide. Across vegetation zones, chloro-lichens that have Trebouxia sp. as photosynthetic partner occupy a wide range of ecosystems with the temperature regimen differing from polar region habitats. Therefore, the genus Trebouxia should be classified rather psychrotolerant than psychrophilic.

Simultaneous measurements of optical density (OD) and \( \Phi_{\text{PSII}} \) during the cultivation of the culture allowed to analyze their relationship. When cultivated at 10°C, Trebouxia showed absolute maximum of growth rate at the same time, at which an increase in \( \Phi_{\text{PSII}} \) values was recorded (see Fig. 2, Table 3, 400–420 h of cultivation). Such increase in the effectiveness of photosynthetic processes in photosystem II is thus responsible for an acceleration of Trebouxia culture growth. This conclusion can be supported by the two facts: (1) No such increase in \( \Phi_{\text{PSII}} \) was found at higher cultivation temperature (15°C) simultaneously with no change in OD, (2) whenever \( \Phi_{\text{PSII}} \) of Trebouxia cultured at 10°C reached constant value after the previous increase (see Fig. 2, 480 h), OD became constant indicating that the culture reached maximum density. Generally, the time required for reaching final density of Trebouxia culture was much longer (840 h) than reported for majority of temperate unicellular freshwater (e.g. Chlamydomonas reinhardtii in Morlon et al. 2005 – 120 h) and marine alga (e.g. Chlorella minutissima in Sankar et al. 2011 – 350 h).

**Concluding remarks**

In this study, we support the idea that Trebouxia sp. isolated from Antarctic lichen Usnea antarctica could be ranked among psychrotolerant species. Recent knowledge on temperature dependence of particular photo- and biochemical processes of photosynthesis in Trebouxia from polar regions is, however, still inadequate. Therefore, axenic cultures of Trebouxia sp. grown in liquid medium represent an excellent tool to study Trebouxia resistance to low temperature and freezing stress (Hájek et al. 2012). Our further study (Barták et al., MS in prep.) will be focused on inhibition of photochemical processes in photosystem II in isolated Trebouxia sp. in relation to gradual cooling of the culture from physiological to freezing temperature.
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