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Pilot cultivation of the green alga *Monoraphidium* sp. producing a high content of polyunsaturated fatty acids in a low-temperature environment

Tomáš Řezanka [a,*], Linda Nedbalová [b,c], Jaromír Lukavský [c], Antonín Střížek [b], Karel Sigler [a]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Charles University, Faculty of Science, Department of Ecology, Viničná 7, 128 44 Prague 2, Czech Republic
[c] Institute of Botany, Academy of Sciences of the Czech Republic, Department of Plant Ecology and Biorefinery Centre of Competence, Dukelská 135, 379 82 Třeboň, Czech Republic

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ABSTRACT

A cold-adapted strain of *Monoraphidium* (Selenastraceae, Chlorophyta) isolated from an Antarctic ice-covered lake was tested for polyunsaturated fatty acids (PUFAs) production. The strain was successfully cultivated in an outdoor 150 L thin-layer photobioreactor under early winter conditions of Central Europe, where the average temperature was 10.0 °C and the average intensity of PAR 32 μmol m^{-2} s^{-1}. The growth rate over the 22 days of the pilot cultivation reached a value of 0.341 day^{-1} and lipid productivity was 162 mg L^{-1} day^{-1}. The proportion of the 16:4 and 18:4 acids was up to 19.1% and/or 34.7% of total fatty acids that resulted in a productivity of these acids of 27.5 and 43.7 mg L^{-1} day^{-1}, respectively, which is one order of magnitude higher than previously reported values. These characteristics make this strain a prospective candidate for low-temperature biotechnology.

1. Introduction

Microalgae have great potential for applications in biotechnology due to their high growth rate and the possibility of industrial mass cultivation in suspension cultures, which are environmentally safe and technically easy to control. In addition, they can be grown in areas unsuitable for conventional crops requiring adequate soil. Unicellular algae have been tested as potential producers of valuable substances, e.g. polyunsaturated fatty acids (PUFAs). These essential compounds are needed as a supplement to a healthy human diet as well as a supplement to fish feed in aquaculture or zooplankton [1,2].

Prominent among the PUFAs are acids with 18 carbon atoms, i.e., linoleic and α-linolenic acids, which are among the essential PUFAs and have no substitute in the diet of vertebrates. These acids are not only used as an energy source, but they are crucial for a number of biological processes in the body. They function as substrates necessary for the synthesis of prostaglandins and other biologically active substances (thromboxanes, prostacyclins, etc.). In terms of the chemical composition, only two fatty acids are primarily essential: α-linolenic acid (polyunsaturated ω-3 fatty acid) and linoleic acid (polyunsaturated ω-6 fatty acid). The other fatty acids, which are referred to as "conditionally essential", include most PUFAs, e.g., stearidonic acid (all-cis-6,9,12,15-octadecatetraenoic acid, 18:4ω–3), which is biosynthesized from α-linolenic acid by Δ6-desaturase. The rare natural sources of this acid are vegetable oils, e.g. seeds of some species from the Boraginaceae family (*Lithospermum*, viper's bugloss, i.e., *Echium*), hemp or black currant. However, the richest sources are photosynthetic microorganisms, particularly algae, in which the content of the acid can be up to several tens of percent [3]. The known sources of all-cis-4,7,10,13-hexadecatetraenoic acid (16:4ω-3) are solely algae, either marine or freshwater [3]. Both 18:4ω-3 and 16:4ω-3 fatty acids from the marine alga *Undaria pinnatifida* inhibit the production of eicosanoids in murine mast cells, particularly leukotriene B4 (LTB4), leukotriene C4 (LTC4) and 5-hydroxyeicosatetraenoic acid (5-HETE) [4].

An organism suitable for outdoor cultivation must easily grow in a given facility, must have a certain resistance to technological treatment (e.g. pumping), oscillations in temperature and light intensity. New organisms and various types of cultivators are therefore constantly sought and tested. So far, the majority of outdoor mass cultivations have been performed in tropical and subtropical zones, because the commercially used strains are mesophilic or slightly thermophilic. A promising approach is therefore to look for an organism that is able to grow at lower temperatures and radiation, i.e., during cold periods that are not suitable for the growth of conventional microalgae. Such biotechnology at low temperatures could be suitable in temperate regions in winter conditions or during polar summer [5].

Polar habitats are potentially a promising source of microalgal strains for low-temperature biotechnology. Photoautotrophic microorganisms from cold habitats use many adaptation and acclimation strategies to

* Corresponding author.
E-mail address: rezanka@biomed.cas.cz (T. Řezanka).

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cope with their harsh environment, including high content of PUFAs [6,7]. *Monoraphidium* (Selenastraceae, Chlorophyceae) is a genus of coccoid green algae that grows in many freshwater habitats such as lakes, bogs, small ponds or oxbows, but is usually not too abundant. During the Czech research expedition to Antarctica in 2009 (James Ross Island, northeastern Antarctic Peninsula), we isolated a strain *Monoraphidium* sp. from the plankton of a 12 m deep ice covered lake, where this species formed the dominant component of phytoplankton [8,9].

All studies published so far dealing with cultivation of the alga *Monoraphidium* and related genera from the Selenastraceae (for example *Ankistrodesmus*) have focused on the production of biodiesel. Recently, strains from this family were shown to be promising candidates in this field due to high lipid productivity and suitable fatty acid profile [10].

In this study, we used the newly isolated strain *Monoraphidium* sp. CCALA 1094 from Antarctica for a pilot-scale outdoor cultivation. The strain grows in a wide temperature range starting from 1 °C, but the growth is inhibited at temperatures above 20 °C. The requirements for low light intensities reflect its original habitat [9]. We had therefore no problems cultivating this strain in early winter conditions at 50° north latitude. We fully confirmed the generally accepted notion that organisms adapted to low temperature have an increased content of PUFAs [6]; the content of all PUFAs was greater than 60%, the content of 16:4 and 18:4 acids reaching as much as 54% of total fatty acids. Together with relatively high growth rates, this strain eminently is suitable as a source of PUFAs for dietary supplements for livestock and humans.

2. Material and methods

2.1. Organism and pre-cultivation

The strain was isolated in 2009 from an ice covered lake on James Ross Island (northeastern Antarctic Peninsula) by plating on an agar plate with BG11 medium [11] and colony selection. It is deposited in the Culture Collection of Autotrophic Organisms, Institute of Botany, CAS, Dukelská 135, Třeboň (CCALA) as *Monoraphidium* sp. CCALA 1094, Nedbalová 2009/1. It is not yet maintained in any other algal collection in the world. Both molecular (18S rDNA, ITS-2 rDNA) and morphological data were used for the identification of the strain. The sequences are available in the NCBI Nucleotide Sequence Database under accession numbers KX671911 and KX671913. Cell morphology corresponded to the characteristics of the genus *Monoraphidium*; however, the strain can hardly be assigned with certainty to any species from the Selenastraceae described so far. Cells were small, spindle shaped, always significantly tapering toward the apices, arcuate with fine rounded ends [9].

Monoraphidium sp. CCALA 1094 is kept as a growing culture on test tube slants at about 15 °C, under fluorescent lighting with photosynthetically active radiation (PAR) intensity of 40 μmol m^{-2} s^{-1}. The culture was used to pre-cultivate the strain in a batch stationary culture in liquid Z8 medium (Table 1S) in 2 L glass bottles containing initially 0.25 L medium, its volume being gradually (after the culture became denser) increased to 2 L. Mixing of the suspension and CO_2 saturation was ensured by bubbling an excess air with 2% CO_2 introduced to the bottom of the bottle via a tube at a flow rate of 350 mL min^{-1}; the gaseous mixture was filtered through a membrane filter (Midisart® 2000 PTFE, porosity 0.2 μm, Sartorius, Germany). The bottles were placed in a water bath and kept at 10 °C by a compressor cooling circuit. The bath was continuously illuminated from the flanks through a glass wall by LED panels with the PAR intensity of 160 μmol m^{-2} s^{-1}. For the first five days of the cultivation and after each volume increase, the light intensity was decreased to 80 μmol m^{-2} s^{-1} until the culture became visibly denser.

2.2. Pilot cultivation

Monoraphidium sp. CCALA 1094 was pilot-cultured on a BCS Engineering Brno open thin-layer photobioreactor (Fig. 1) located in a temperature uncontrolled greenhouse of the Institute of Botany, CAS, in Třeboň, Czech Republic (49°0′21.546″N, 14°46′21.538″E). It has a glass sloping surface of 12 × 1 m, height gradient 15 cm, volume 150 L. This photobioreactor type is intended for very thick slurries in a thin layer (up to 10 mm) and it was developed at the Institute of Microbiology, CAS, in 1963 and its modified version (from 1991) has been used for commercial production of algal biomass. For further technical details see [12].

The inoculum was prepared as described above and its whole volume was transferred to 150 L of unsterilized Z8 medium. The initial concentration of algae in the open photobioreactor after inoculation was 0.5 g L^{-1} dry weight (DW). Dry biomass of the sample was determined by a 20 min centrifugation at 3000*g* in pre-weighed plastic 2 mL Eppendorf tubes. The sediment was dried at 105 °C to constant weight and weighed.

CO_2 was dosed into the medium by a centrifugal pump for rapid absorption by algal suspensions and its concentration was regulated at 5 L·h^{-1}. Tap water was added several times during the cultivation to compensate the losses due to evaporation. The experiment was run from 26 November to 17 December 2015. Throughout the cultivation, the temperature of the suspension and PAR intensity inside the greenhouse were recorded at ten-minute intervals using Minikin data loggers (EMS, Brno, Czech Republic). The culture growth was monitored daily by measurements of the optical density at 750 nm (OD_{750}). The OD_{750} values were converted into cell density (N, cells mL^{-1}) using the

Table 1
The content of fatty acids and lipids during the pilot outdoor cultivation of *Monoraphidium* sp. CCALA 1094, the samples were harvested on the day 7, 14 and 22 of the experiment (n = 3 measurements).

Fatty acid	Common name	Harvested		
		3.12. (% ± S.D.)	10.12. (% ± S.D.)	17.12. (% ± S.D.)
13:0	Tridecanoic[a]	0.8 ± 0.2	0.9 ± 0.3	0.5 ± 0.2
14:0	Myristic	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
16:0	Palmitic	17.0 ± 0.8	17.4 ± 1.5	17.5 ± 1.0
16:1ω7	Palmitoleic	3.5 ± 0.4	2.5 ± 0.2	1.9 ± 0.5
16:1ω13	Hexadecenoic[a]	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
16:3ω3	Hexadecatrienoic[a]	1.7 ± 0.5	1.7 ± 0.8	1.8 ± 0.8
16:4ω3	Hexadecatetraenoic[a]	19.1 ± 1.4	18.4 ± 0.9	17.0 ± 0.9
18:0	Stearic	4.3 ± 0.6	3.2 ± 0.4	1.7 ± 0.4
18:1ω9	Oleic	10.1 ± 1.1	12.5 ± 1.3	17.0 ± 1.9
18:1ω7	Cis-vaccenic	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
18:2ω6	Linoleic	2.3 ± 0.7	4.0 ± 0.6	5.7 ± 0.7
18:3ω6	γ-Linolenic	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.2
18:3ω3	α-Linolenic	4.9 ± 0.8	6.4 ± 0.8	7.9 ± 0.7
18:4ω3	Stearidonic	34.7 ± 1.4	31.8 ± 1.7	27.5 ± 1.3
22:0	Behenic	0.9 ± 0.2	0.8 ± 0.3	0.5 ± 0.2
Lipids per dry weight (%)		33.6 ± 0.7	24.5 ± 0.5	27.0 ± 0.6

[a] Systematic names.

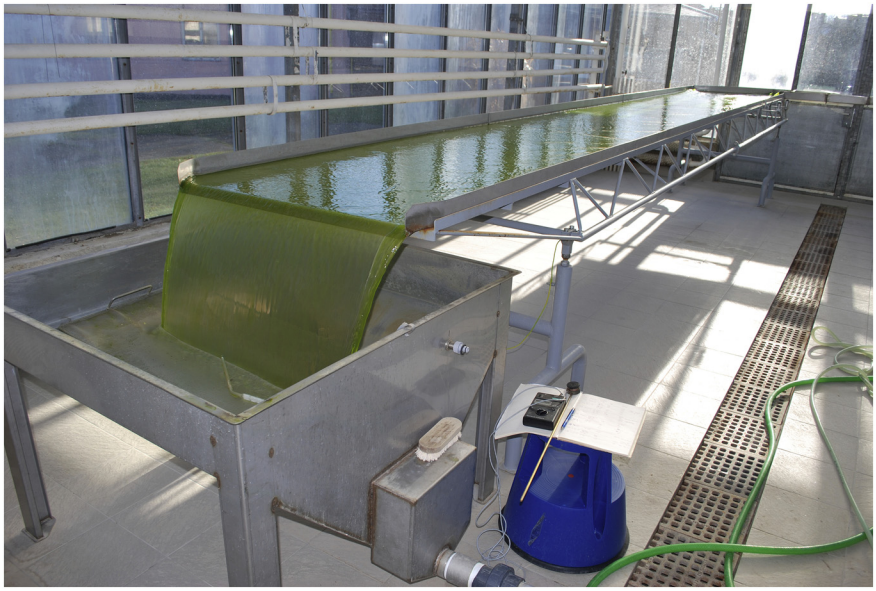

Fig. 1. View of the open thin-layer photobioreactor containing a culture of *Monoraphidium* sp. CCALA 1094 after 10 days of cultivation.

following equation obtained from the conversion curve between the two parameters:

$$N = \frac{OD_{750} - 4.41^{-2}}{7.27 \times 10^{-9}}$$

The specific growth rate (μ, day^{-1}) was calculated according to the equation:

$$\mu = (\ln N_f - \ln N_i)/(t_f - t_i)$$

where N is the cell density at final (f) or initial (i) time (t). The maximal specific growth rate (μ_{max}) was the highest μ recorded throughout the cultivation.

After 22 days the biomass was harvested by centrifugation in an EVODOS 10 centrifuge (at 7000 rev min^{-1}), frozen to −20 °C, lyophilized (vacuum freezing at a pressure of 0.05 hPa) and weighted.

2.3. Lipid content and identification of fatty acids

The samples for analysis of lipid content and FA composition were taken on days 7, 14 and 22 of the experiment. Each sample was measured three times. The extraction procedure was based on the method of Bligh and Dyer [13] except that 2-propanol was substituted for methanol, since 2-propanol does not serve as a solvent for phospholipases [14]. The alcohol-water mixture was cooled from room temperature to 4 °C, one part of chloroform was added and the lipids from the lyophilized cells were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts of 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure and total lipids were weighed, see Table 1.

10 mg of total lipids were saponified with 10% KOH solution in methanol at room temperature overnight. Neutral or basic compounds in a solution of pH 9 were extracted with diethyl ether, the aqueous solution of fatty acids was acidified to pH 2 and the acids were extracted into hexane. Fatty acids were methylated with a BF_3-methanol mixture and identified by GC–MS (gas chromatography with mass spectrometry, ion trap detector with electron impact; Finnigan 1020 B, Finnigan, San Jose, CA, USA). The sample was injected into a capillary column with polar stationary phase, 25 m × 0.25 mm × 0.1 μm (Supelcowax 10; Supelco, Prague, Czech Republic) and eluted for 5 min at 50 °C. The column was then heated at a rate of 10 °C min^{-1} to 230 °C and was then operated isothermally for 15 min at 230 °C. The carrier gas was He with a flow rate of 0.52 mL min^{-1}. All the spectra were scanned in the range of 50–600 Da. The structure of methyl esters was determined based on the retention times and by comparing their fragmentation spectra with the spectra of pure compounds (PUFA No.1 (Marine Source, Cat. No. 47033) from Sigma-Aldrich, Prague, Czech Republic).

3. Results and discussion

3.1. Culture growth and weather conditions

Cell densities of the strain *Monoraphidium* sp. CCALA 1094, the temperature profile of the medium and the intensity of PAR during the whole cultivation period are shown in Fig. 2. Maximum specific growth rate (μ_{max}) was 0.856 day^{-1}, the specific growth rate throughout the pilot cultivation was 0.341 day^{-1}. This is consistent with the laboratory experiment that was performed in a cultivation unit for crossed gradients, where the maximum growth rate of this strain was 0.5 day^{-1} [9]. The growth curve showed a rather long lag phase probably due to extremely low light intensity, a rapid transition to a stationary phase and then restart of growth that lasted until the end of the experiment. After 22 days of cultivation the culture attained a final density of 11×10^8 cells mL^{-1}, which was 13.6 g L^{-1} DW (the total harvested amount was 2035 g DW, i.e., 169.6 g m^{-2}, or 7.7 g m^{-2} day^{-1}, or 0.6 g DW L^{-1}·day^{-1}). Microscopic analysis showed that the strain can tolerate mechanical stress caused by the centrifugal pump; the portion of mechanically broken cells was below 1%. The temperature of the slurry ranged from 3.2 to 21.9 °C, average ambient temperature over the whole period was 10.0 °C. PAR values ranged from 0 to 726 μmol m^{-2} s^{-1}, the average intensity of PAR being a mere 32 μmol m^{-2} s^{-1}.

The biggest advantage of the strain CCALA 1094 was the opportunity to cultivate it at low temperatures and irradiance. Since the alga was isolated in Antarctica, i.e., at a site situated at latitude 64° South, it was reasonable to assume that the alga will be, if not psychrophilic, then at least psychrotolerant. These assumptions were fully confirmed during a previous laboratory experiment in a cultivation unit for crossed gradients. The strain was able to grow in a wide temperature range (1–20 °C) and the highest growth rates were detected in the range from 6 to 20 °C [9]. The cultivation temperature in outdoor conditions during the pilot cultivation naturally fluctuated around this value. Only one

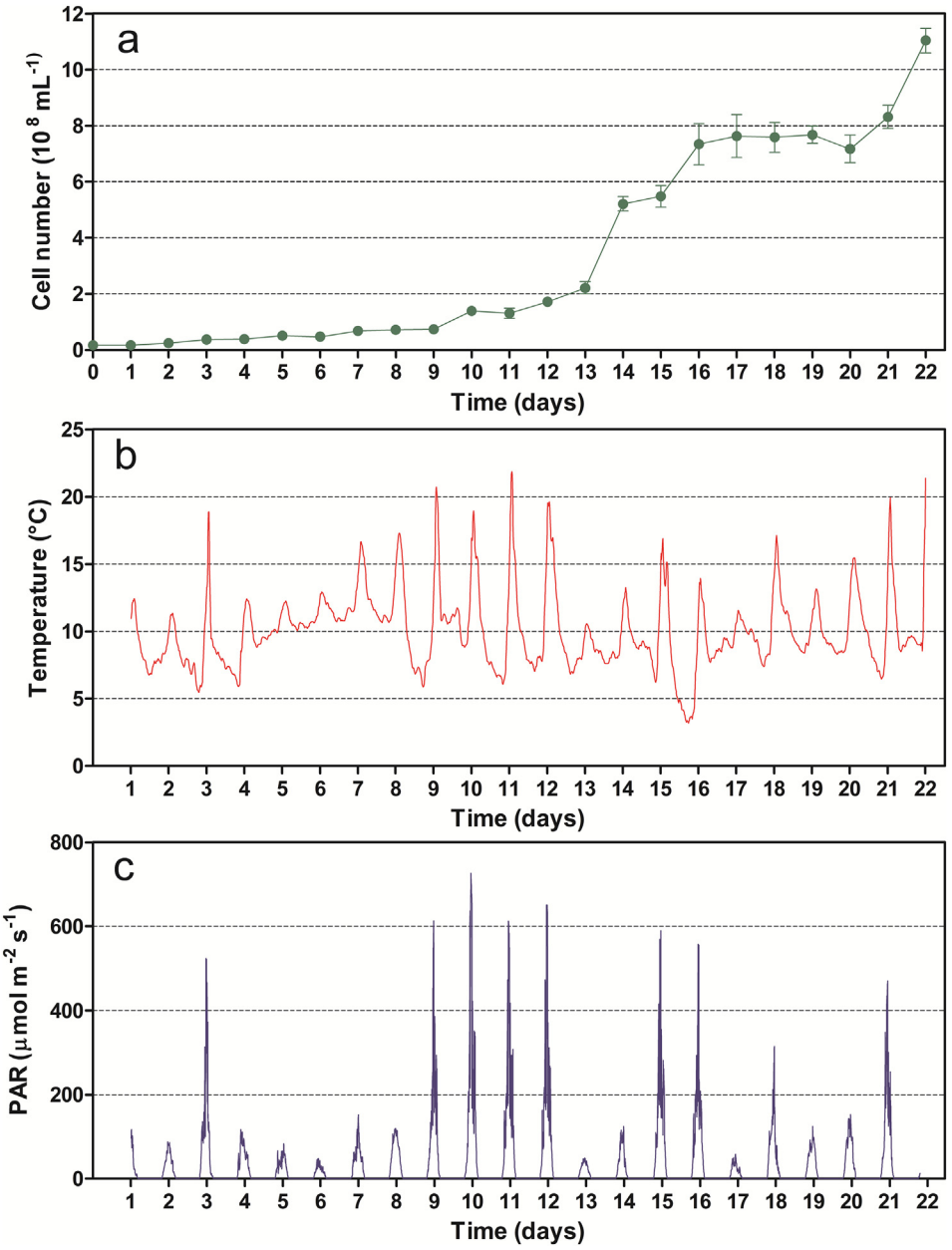

Fig. 2. Time course of the growth curve (±SD, n = 3) (a), temperature of the culture medium (b) and the PAR intensity (c) in outdoor cultivation on a platform.

work [15] out of more than 20 studies mentioned in a review by Yee [10] focused on *Monoraphidium* strains used a cultivation temperature below 20 °C, in all other cases the algae were grown mostly at or above 25 °C [10]. Obviously, the further away from the equator, it is more likely to isolate strains with lower temperature optima for growth. Holbrook et al. [15] used for their cultivation *Monoraphidium* isolated from a greenhouse near Chicago (about 42° north latitude), but to optimize the cultivation they still used both air cooling and heating in the greenhouse.

In the crossed gradients experiment not only the optimal temperature for growth was determined but also the optimal irradiance, since the light conditions at the collection site are poor (thick ice cover preventing light penetration combined with high latitude). The optimum irradiance was ~20 µmol m^{-2} s^{-1} [9], which is close to the average value observed during the outdoor cultivation. None of the strains reviewed in [10] has so far been cultivated at such low values. To sustain sufficient growth rates, strains of *Monoraphidium* and other green coccoid algae (*Ankistrodesmus* and *Kirchneriella*) were cultivated at irradiation levels several times higher (60 to several hundred µmol m^{-2} s^{-1}). The low light and temperature demands make *Monoraphidium* sp. CCALA 1094 a good candidate for outdoor cultivation in temperate regions in winter conditions or during polar summer, as confirmed by this study. The growth rate and biomass productivity was comparable or even superior to values from experiments performed at significantly higher temperatures and higher irradiances [16,17].

3.2. Lipid and PUFAs production

In the first phase of the pilot cultivation, total oil content in the biomass of *Monoraphidium* sp. CCALA 1094 was 33.6 + 0.7% in DW, the PUFAs comprised 62.8 ± 0.8% of total fatty acids. Both parameters slightly declined in the course of cultivation (Table 1). For this reason the experiment was terminated after 22 days even though the culture continued to grow (Fig. 2). In such outdoor experiments with uncontrolled environmental parameters, it is difficult to discern with certainty the causes of these changes. The decline in total lipid and PUFA content

between the first and second measurement could be due to a slight increase in light intensity (average PAR intensity was 48 μmol m^{-2} s^{-1} in this period) and related increase in growth rate (Fig. 2). It is generally accepted that starvation can induce higher oil and PUFA content in microalgae [18]. Therefore, it could also explain that the lipid and PUFA content in the strain CCALA 1094 was highest in the first phase of the outdoor cultivation, that was characterised by slow growth.

In the genus *Monoraphidium*, the lipid content in DW was reported from 19.9 to 43.5% [10]. The values obtained during the outdoor cultivation of strain CCALA 1094 ranged from 24.5% to 33.6% depending on the length of cultivation. This places the strain near the average. Far more interesting for biotechnological applications, however, is the value of the lipid productivity; the data from the literature give values of up to 90 mg L^{-1} day^{-1} for *Monoraphidium* strains, only study [19] gives a value between 140 and 190 mg L^{-1} day^{-1}. The value detected during the cultivation of the strain CCALA 1094 was 162 mg L^{-1} day^{-1}, which exceeds the average productivity reported for other *Monoraphidium* strains (~60 mg L^{-1} day^{-1} [e.g., 15–17, 20,21]).

The preference of low temperature and irradiance [9] makes the strain CCALA 1094 unique also within the Selenastraceae [10] and this is reflected also in its fatty acid profile. The proportion of the 16:4 and 18:4 acids was up to 19.1% and/or 34.7%, respectively. In Fig. 3, the content of these two acids is compared to the data presented by Lang et al. [3] and also in [15–17] and [19–32]. None of the other studies describing the content of PUFAs in the genus *Monoraphidium* and related genera (see above) reported such a high content of C16 and C18 PUFAs.

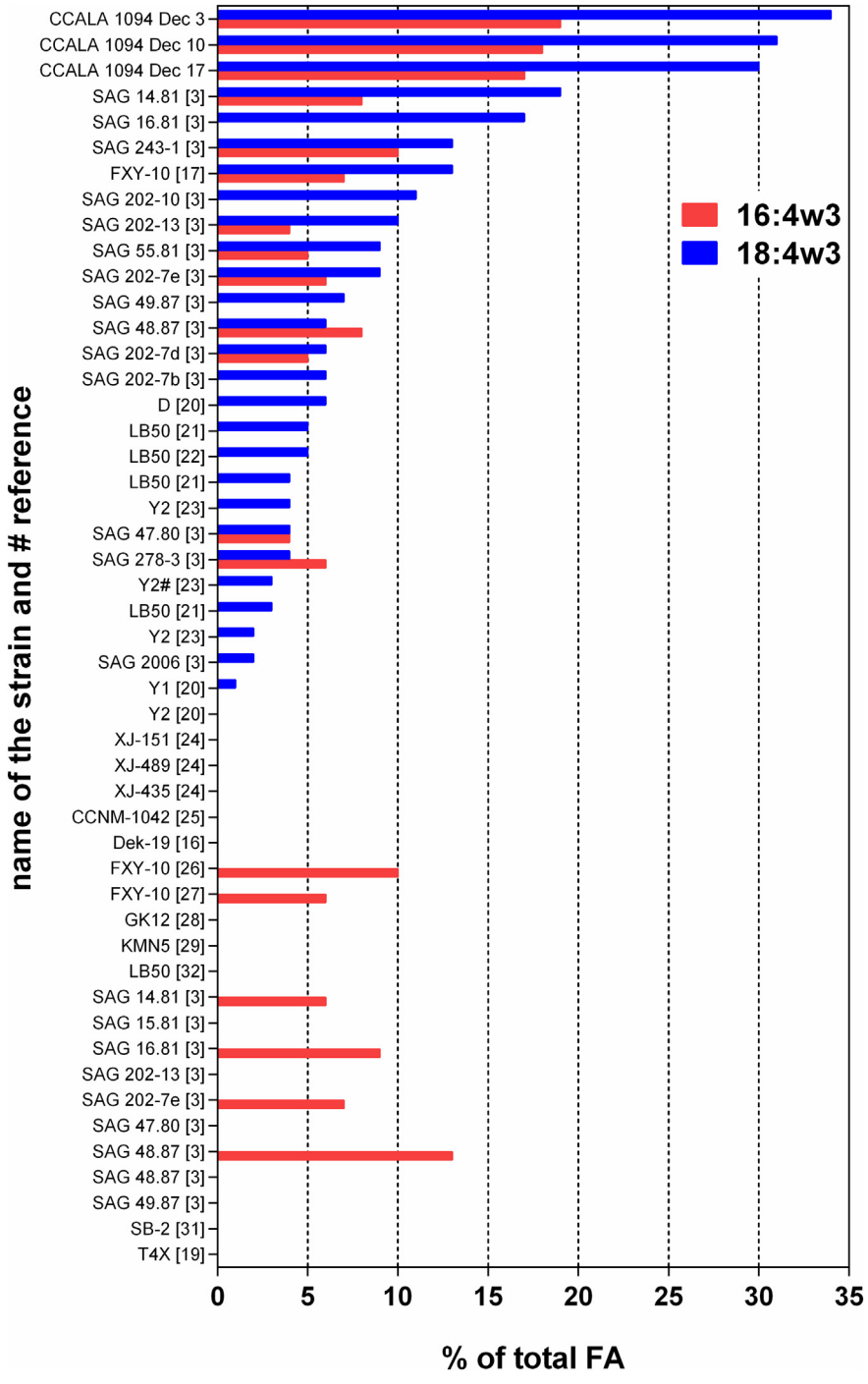

Fig. 3. Production of 18:4ω-3 and 16:4ω-3 fatty acids by *Monoraphidium* sp. strain CCALA 1094 and other *Monoraphidium* strains (the reference number is listed for each strain).

A major problem in the interpretation of results concerning the fatty acid profiles is that the values published in many articles often vary widely. While for example, according to [3] the content of 16:4 and 18:4 acids in strain SAG 14.81 was around 8.9 and 19.1% of total fatty acids, respectively, Bogen et al. [16] using GC–MS did not detect the 18:4 acid in the strain SAG 14.81 at all. A similar situation can be observed with another strain of *Monoraphidium*, i.e. FXY-10, in which Zhao et al. [17] found the 16:4 and 18:4 acids in an amount of about 7.6% and 8.1% of total fatty acids, respectively, whereas other studies performed by [26] and also [27] do not mention the presence of the 18:4 acid. The difference between the content of PUFAs and particularly between the contents of the two tetraenoic acids (16:4 and 18:4) in the alga *Monoraphidium* cited in the literature and our results may be due to the fact that the analyzed species and strains of *Monoraphidium* were isolated from very different habitats (i.e., they are probably geographical varieties). Furthermore, a very significant factor influencing the content of PUFAs is the cultivation temperature; except for a single article [15] all studies cited in review [10] used a cultivation temperature above 20 °C. The representation of PUFAs, and specifically tetraenoic fatty acids, may therefore vary depending on environment/origin of algae and cultivation temperature. In any case, the content of 16:4 and 18:4 acids in the strain CCALA 1094 exceeds several times the published contents of these acids in most *Monoraphidium* strains, see Fig. 3.

Therefore, the properties of strain CCALA 1094 come to the fore if we take into account not only lipid productivity but also the productivity of PUFAs, which was 97.7 mg L^{-1} day^{-1} and, when calculating the yield per m^2, 1.26 g m^{-2} day^{-1}. While higher values were achieved only in laboratory closed cultivation systems, our equipment, though situated in a greenhouse, was of the open flatbed cultivator type. The productivity of 16:4 and 18:4 acids reported by [17] was 1.4 and 2.5 mg L^{-1} day^{-1} whereas in the strain CCALA 1094 the productivity of the two acids was one order of magnitude higher, i.e. 27.5 and 43.7 mg L^{-1} day^{-1}, respectively.

4. Conclusion

It appears that because of the low requirements for cultivation temperature and a minimal requirement of light, our strain is biotechnologically very promising, compared to other strains of this genus; its production of PUFAs, particularly 16:4 and 18:4, is several times higher than previously reported values. *Monoraphidium* sp. CCALA 1094 is thus a prospective new organism for use in low-temperature biotechnology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.12.017.

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