



The alga *Bracteacoccus bullatus* (Chlorophyceae) isolated from snow, as a source of oil comprising essential unsaturated fatty acids and carotenoids

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Abstract

A unicellular alga isolated from snow in the Sierra Nevada Mountains (Spain) was characterised using a polyphasic approach. Comparative analysis of ITS2 rDNA secondary structures identified the new culture (CCALA 1120 Cepák and Lukavský Nova Hedwigia 94:163–173, 2012) as being conspecific with *Bracteacoccus bullatus* (Chlorophyceae). For the first time this study documented sexual reproduction as the pairing of gametes and also an-isogamy. Strain CCALA 1120 had a temperature optimum of growth about 21 °C and an irradiance optimum above 160 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. It was cultivated in pilot-plant scale, using an open thin-layer photobioreactor in a greenhouse with only partial temperature control. After harvest, a high proportion of fatty acids was found (15.3% of dry mass) with linoleic (18:2 ω -6) 18.3% and α -linolenic acids (18:3 ω -3) 17.4% being the most abundant. Monounsaturated fatty acids accounted for about 30% with oleic (18:1 ω -9) and vaccenic acids (18:1 ω -7) as the most prominent. The ratio of PUFA ω -6/ ω -3 was 1:1.16, i.e. near to the ideal ratio of 1:1, as recommended by the World Health Organization. Biomass production was 2.67 g $\text{m}^{-2} \text{day}^{-1}$ of dry weight, i.e. 0.2 g $\text{L}^{-1} \text{day}^{-1}$. At the end of growth phase, total carotenoids made up 10.1 mg L^{-1} . These results indicate that *B. bullatus* is suitable for production of a vegetable oil at lower temperatures (12–18 °C) and comprising a high content of unsaturated fatty acids.

Keywords *Bracteacoccus bullatus* · Cryoseston · Polyunsaturated fatty acids · Carotenoids · Pilot plant cultivation · Biotechnology · Molecular taxonomy

Introduction

Essential fatty acids in the diet are necessary for the healthy functioning of organisms because humans, as well as other mammals, are unable to synthesize them. Strictly speaking, there are only two primary essential polyunsaturated fatty acids (PUFAs): ALA (α -linolenic, 18:3 ω -3) and LA (linoleic, 18:2 ω -6) (Nakamura and Nara 2003). Two groups of PUFAs are distinguishable, ω -3 and ω -6. The former is represented for instance by ALA and hexadecatrienoic acid (HDA; 16:3 ω -3). The latter include, in particular, LA and γ -linolenic (GLA; 18:3 ω -6). ALA belongs to so-called drying-capable vegetable oils. The source of HDA is exclusively photosynthetic microorganisms, mainly marine or freshwater algae, where the content reaches up to tens of percent. These essential substances are necessary as a feed supplement for fish in aquaculture, or zooplankton, which then serves as food for fish (Weylandt et al. 2015).

Oleic acid (OL; 18:1 ω -9) occurs in various animal fats and vegetable oils. It is the precursor to the synthesis of essential

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ALA. PUFAs are the main factor in the favourable influence of the so-called Mediterranean diet. For vegans, vegetarians and for those who lack fish in their diet, these algal and plant products represent an acceptable alternative (Cherif et al. 1975).

Algae, as a source of FAs, e.g. *Nannochloropsis oceanica*, *N. limnetica* and *Lobosphaera incisa*, have potential for use in biotechnology because of their high growth rate and the possibility of automated large-scale cultivation. In addition, they can be grown in locations unsuitable for conventional crops because they do not require fertile soil, e.g. in deserts, on roofs of industrial buildings, etc. (Krienitz and Wirth 2006; Xiao et al. 2013; Siegler et al. 2017). Outdoor cultivation eliminates the costly supply of heat and light energy. In a temperate climate, however, it is possible to operate outdoor cultivation of algae only for a limited period of the year, when the air temperature and solar radiation is high enough for the growth of these microorganisms (Borowitzka 2013). Therefore, there is demand for new organisms with advantageous properties (Lang et al. 2011), e.g. snow algae (Hoham and Remias 2020). Some strains of algae can be used for the production of oils containing high levels of PUFAs, e.g. *Monoraphidium* sp. for the production of HDA and stearidonic acid (Řezanka et al. 2016, 2017). Temperature dependence of production of structured triacylglycerols in the alga *Trachydiscus minutus* (Řezanka et al. 2015) may be given as examples, too.

However, the correct proportion of ω -6/ ω -3 PUFAs is most important and excess is not beneficial for health (Cunnane 2003). Examples of native ratios of ω -6 and ω -3 PUFAs in biotechnologically cultured algae were reported by Lang et al. (2011): for commonly used algae such as *Chlorella* sp., the ratio of ω -6/ ω -3 was 1: 0.40, *Parachlorella kessleri* (formerly *Chlorella kessleri*) 1: 0.19, *Scenedesmus* sp. 1: 0.45. In cyanobacteria, the ratio of these FAs varied from 1: 0.30 by *Arthrospira* (*Spirulina*) to cases where cyanobacteria did not produce any ω -3 PUFAs.

Bracteacoccus has been investigated in the bioprospecting of microalgae for biofuel production (Piligaev et al. 2015; Isaac 2020) and *B. cinnabarinus* was shown to grow heterotrophically in medium supplied with sodium or potassium acetate and glucose (Hornung et al. 1977). Subsequently, for *B. bullatus*, a tenfold reduction in phosphorus and nitrogen in the nutrient solution resulted in OL, LA and palmitoleic acid (PA; 16:1 ω -7) levels reaching 48–64%, 14–24% and 9–13% of total FAs, respectively. The latter alga was cultivated at laboratory scale under constant shaking and saturated with 5% CO₂, under irradiance of 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16/8 h photoperiod (Mamaeva et al. 2018). Maltsev et al. (2020) isolated, a new strain of *B. bullatus* (MZ-Ch32) from soil that produced dry biomass and tFAs to 2.31 g. L⁻¹ and 55.84%, after 15 days of cultivation. In the total fatty acids, palmitic, hexadecadienic (12.5%), oleic (43.2%) and linoleic acids (23.8%) prevailed. A balanced ratio of ω -6/ ω -3 PUFAs

makes the strain prospective as food additives and high content of oleic acid for the biofuel production.

About 1100 carotenoids are known (Yabuzaki 2017), the most frequently produced commercially is β -carotene and astaxanthin, whose industrial production is derived from plant- or animal-based and synthetic sources. Natural carotenoids are key components of functional food cosmetics, drugs and animal feeding (Chekanov et al. 2021). The main algal source is *Haematococcus lacustris* (*H. pluvialis*) and *Dunaliella salina* (Borowitzka 2013; Rajesh et al. 2017). Astaxanthin is also found in yeasts, salmon, trout, krill, shrimps, crustaceans, and feathers of some birds, majority is synthesized chemically (Chekanov et al. 2021).

Bracteacoccus minor has previously been shown to be a promising producer of carotenoids, including astaxanthin diesters comprising 37–42% of total carotenoids, and 53–63% of lipids in dry matter. In this case, a two stage cultivation process was performed, the first (green, 16 days) followed by a second one (red, 11 days, diluted to reduce nitrogen and phosphorus levels), and astaxanthin production was also stimulated by spiking with Na-acetate (Minyuk et al. 2014). Chekanov et al. (2021) has obtained very promising data with *B. aggregatus*.

The aim of the current work was to test an isolate of microalga from an extreme mountain snow habitat in order to acquire a producer of valuable fatty acids and carotenoids. The strain should be capable of growing at lower temperatures and light intensities, and thus be adapted to conditions in open pond reactors.

Methods

Sampling site and isolation of algal strain

A green microalga was isolated by J. Lukavský from snow samples collected by V. Cepák in Sierra Nevada (Spain) during the course of studying the diversity of cryoflora (Cepák and Lukavský 2012). The strain was deposited to the Culture Collection of Autotrophic Organisms of Inst. Botany Acad. Sci. Třeboň, Dukelská 135, under No. CCALA 1120.

Pre-cultivation and pilot cultivation

The strain was pre-cultivated in the laboratory, from a test tube slant culture transferred into 2L bottles with nutrient solution after Zachleder et Šetlík (1982), aerated by excess filtered (Millipore, Midisart 2000, PT FE IN, pores 0,2 μm) air with 2% CO₂ and irradiated by a white LED panel of light intensity 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (although the first 2–3 days were supplied with only 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The bottles were placed in a water bath at a temperature of 7–10 °C. After

pre-cultivation, the inoculum was transferred into a pilot-plant cultivation unit (BSC Engineering Brno, CZ), of volume 150 L, area 12 m², length 12 m, thickness of suspension 10–15 mm, located in a greenhouse (Doucha and Lívanský 1996, Supplementary Fig. S1). This unit had a surface to volume ratio of 80 (Venancio et al. 2020). Three cultivation periods were performed: April 4–June 9 2016, March 23–May 1 2017, and December 7 2018–April 17 2019. The green house was heated only when the temperature dropped below 8 °C. Nutrient solution (Zachleder and Šetlík 1982) was diluted to ½ with tap water, CO₂ was supplied only during the day, at a pumping rate of about 5 L·min⁻¹. Air and suspension temperatures, as well as PAR (photosynthetically active radiation), were recorded continuously at 10 min intervals by Tie and QTl dataloggers (Minikin EMS, Brno, CZ). Growth curves were evaluated every day by absorbance at 750 nm in a spectrophotometer (Shimadzu UV-1800 PC, Japan) in 1 cm cuvettes (after dilution < 0.8 Abs₇₅₀). The initial phase of strain cultivation comprised steps with excess nutrients for production of biomass, while the final step was carried out without nutrients to increase lipid production. Biomass was harvested by centrifugation by EVODOS 10 (Evodos BV, Netherlands) at 4200 rpm, 3000 × g, frozen to -20 °C and lyophilized under 0.05 hPa (Gregor Instr., CZ). Dry weight was evaluated by centrifugation for 20 min at 3000 × g, in pre-weighed Eppendorf tubes (2 mL), and dried at 105 °C to a constant weight.

Temperature and light growth requirements

The experimental strain, CCALA 1120, was cultivated in crossed gradients of temperature and light in the same manner as in previous reports (Kvíděrová and Lukavský 2005), but in Petri dishes of volume 20 mL. The strain was grown in a temperature gradient ranging from 11 to 26 °C and irradiance gradient spanning from 4 to 160 μmol photons m⁻² s⁻¹. A total of 35 combinations of 7 different temperatures and 5 irradiances were used. The biomass yield, expressed as dry weight, was evaluated gravimetrically at the end of the cultivation, as described above, but using the whole volume i.e. 20 mL of suspension.

Analysis of fatty acids

For identification of fatty acids, 100 mg of all freeze-dried biomass samples were soaped with a 10% solution of KOH in methanol, overnight at room temperature. Neutral and basic compounds were isolated from a solution at pH 9 by shaking with diethyl ether and the aqueous solution containing fatty acids was acidified to pH 2; the fatty acids were subsequently extracted into hexane. Fatty acids were

methyated using a mixture of BF₃-methanol and identified using GC-MS, i.e., gas chromatography—mass spectrometry, with an ion trap to detect ionization collision of electrons. The sample was injected into a capillary column with a polar stationary phase, 25 m × 0.25 mm × 0.1 μm and elution was carried out using a temperature gradient of 5 min at 50 °C, followed by heating the column at a rate of 10 °C min⁻¹ to 240 °C and then isothermal for 15 min at 240 °C. The carrier gas was helium with a flow rate of 0.52 mL min⁻¹. All spectra were scanned in the interval from *m/z* 50 to *m/z* 600 in electron impact mode (70 eV). Structures of methyl esters were determined on the basis of retention times, their fragmentation, and by comparison of mass spectra with those of commercially obtained standards.

Analysis of pigments

Total pigment concentrations: All chemicals were a.g. from Sigma-Aldrich, organic solvents were from Analytika (CZ), all solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, CZ). Photosynthetic pigments, including carotenoids, were extracted by homogenizing samples in 100% methanol and then filtration through glass fibre filters. Spectra of the resulting extracts were recorded on a Shimadzu 2600 spectrophotometer (Japan), and chlorophyll *a*, chlorophyll *b* and total carotenoid concentrations were calculated using the equations described by Lichtenthaler and Wellburn (1983).

HPLC: For detailed pigment analysis, the cells were removed from culture medium by centrifugation for 10 min at 4.500 × g. The sediment was then extracted twice at room temperature for 15 min with 100% methanol and the combined extracts were clarified using 0.2 μm nylon filters (Micro-spin centrifuge filter, Alltech, IL, USA). The extracts were further analyzed by high-performance liquid chromatography (HPLC, Agilent Technologies Inc., USA) and a UV-VIS diode-array detector (Agilent DAD 61315B). Pigments were separated using the method of Heukelem and Thomas (2001) on a thermostatic (35 °C) Phenomenex Luna 3μ C8(2) 100 Å column with binary solvent system (0 min 100% A, 20 min 100% B, 25 min 100% B, 27 min 100% A, 30 min 100% A; A: 70% methanol + 28 mM ammonium acetate, B: methanol). The solvent flow rate was 0.8 mL min⁻¹. The injection volume was 20 μL. Peak assignment was based on comparison of the absorption spectra with the known retention behaviour of carotenoids in a reverse phase system (Lichtenthaler et al. Wellburn 1983, Heukelem and Thomas 2001, Yabuzaki 2017).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted according to Procházková et al. (2018). The 18S small subunit ribosomal RNA gene (18S rRNA), nuclear rDNA internal transcribed spacer

region 1 (ITS1), 5.8S rRNA gene, nuclear rDNA internal transcribed spacer 2 (ITS2) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene sequence (*rbcL*) were amplified from DNA isolates by polymerase chain reaction using existing primers (Supplementary Table S1). Amplification and sequencing reactions for these markers were identical to those described by Procházková et al. (2018). The sequences obtained for strain CCALA 1120 were submitted to GenBank genetic sequence database at the National Center for Biotechnology Information (NCBI) (18S + ITS1 + 5.8S + ITS2: MT856650; *rbcL*: MT863786).

Secondary structure prediction of nuclear ITS2 and phylogenetic analyses

The methods of annotation and prediction of the secondary structure of the nuclear ITS2 region were the same as those described by Procházková et al. (2018). The concatenated 5.8S rDNA-ITS2 alignment contained 23 *Bracteacoccus* spp. sequences (443 bp) examined in previous studies (Fučíková et al. 2012; Mamaeva et al. 2018) as well as sequences of the strain CCALA 1120; *Neochloris aquatica* and *Acutodesmus obliquus* were selected as the outgroup (Mamaeva et al. 2018). The best-fit nucleotide substitution model was estimated by jModeltest 2.0.1 (Posada 2008). Based on the Akaike Information Criterion, the K81uf+I model and GTR+G was selected for 5.8S rDNA and ITS2 rDNA, respectively. The phylogenetic tree based on a combined partitioned (by gene) dataset of 5.8S-ITS2 rDNA was inferred by Bayesian inference (BI) (Huelsenbeck and Ronquist 2001) and maximum likelihood (ML) (Felsenstein 1981) according to Nedbalová et al. (2017), with the minor modification that Markov Chain Monte Carlo runs were carried out for three million generations in BI. Convergence of the two cold chains was checked by the average standard deviation of split frequencies (0.00308 for the 5.8S-ITS2 dataset). Bootstrap analysis (Zwickl 2006) and Bayesian posterior probabilities (Felsenstein 1985) were calculated as described by Nedbalová et al. (2017). Furthermore, ITS2 is hypervariable molecular marker used for species-level taxonomy in eukaryotes (Coleman 2009). The presence of CBC in specific helices of the ITS correlates with reproductive isolation and could thus be used for delimiting species (Wolf et al. 2013). The ITS2 sequences of CCALA 1120 (this study) and *B. bullatus* type strain SAG 2032 (Fučíková et al. 2012) were aligned using the sequence-structure analysis in 4SALE (Seibel et al. 2006, 2008) in order to find compensatory base changes (CBCs; nucleotide change at both of the positions that pair with each other in a double stranded helix). Besides, ITS2 sequence of CCALA 1120 was compared with its closest NCBI hit *B. bullatus* strain KF10 (JQ281851.1; Fučíková et al. 2012) and recently lipidomically investigated *B. bullatus* strain MZ-Ch11 (KY066480; Mamaeva et al. 2018)

as well. The secondary structure of nuclear ITS2 was drawn using VARNA version 3.9 (Darty et al. 2009).

Light microscopy

Microscopic observations of the strains were carried out using an Olympus BX51 microscope equipped with an Olympus DP 71 (Japan) digital camera and an HI 100x/1.35 objective.

Statistical analyses

Statistical analyses were performed using Statistica 13.2 software (Dell 2015) and CANOCO 5 (Ter Braak and Šmilauer 2012). Descriptive statistics were used to analyze the abiotic conditions. RDA (redundancy analysis) was used to test the effects of environmental variables on biomass yield. GAMs (generalized additive models) were used to determine biomass yield optima. The results were considered significant for $p < 0.05$.

Results

Light microscopy

Cells of strain CCALA 1120 (Fig. 1A–K) were spherical, rarely pear-shaped (Fig. 1J), with a diameter of up to 15–17 μm . There were about 10 chloroplasts inside each adult cell, and chloroplasts were disc-shaped, parietal, without a pyrenoid. The nucleus was usually in the centre of the cell. Reproduction was by a large number of zoospores, up to 128 (Fig. 1B, I, J), oval to drop-shaped, with two equal flagella emerging from the apex. Papillae were not prominent, and a small spherical stigma was present in one of the chloroplasts, in the posterior region of the cell (Fig. 1C–G). Two contractile vacuoles were in the apex. Zoospores started to move within the mother cell (Fig. 1B, C). After release from the mother cell wall, these motile stages could be recognized as gametes, able to perform anisogamy (Fig. 1D, E) or isogamy (Fig. 1F). If zoospores were not released, their movement inside the mother cell slowly ceased, flagella were discarded and cells were rounded and turned into aplano-spores. (Fig. 1K). In old cells, a red pigment was produced.

Taxonomic identity of the strain using genetic data analysis

According to the phylogenetic analysis of the 5.8S rRNA-ITS2 rDNA regions, the strain CCALA 1120 was placed into the highly supported *B. bullatus*-clade ('B'; Fig. 2). Concerning *rbcL*, the partial sequence (930 bp) obtained for this marker of CCALA 1120 was identical with the *B. bullatus* strain KF10 (JQ259875). Concerning 18S rRNA

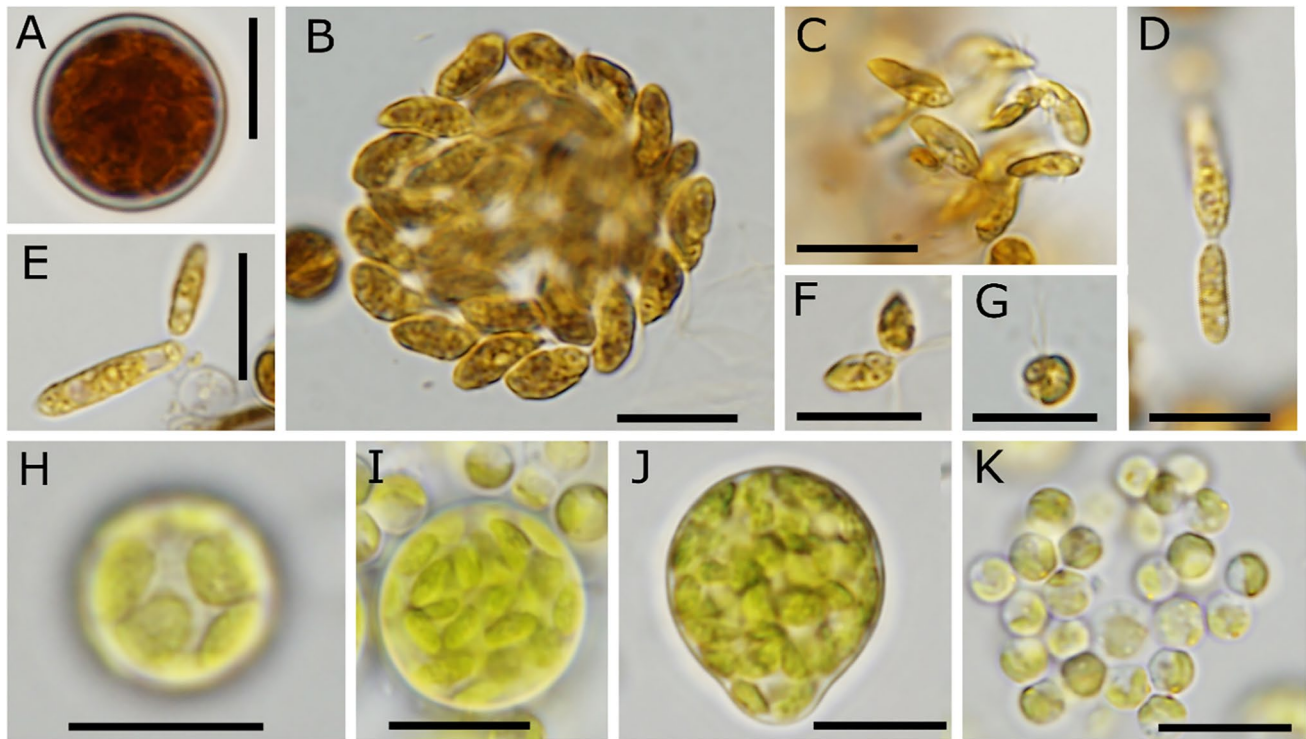


Fig. 1 Life cycle of *Bracteacoccus bullatus* strain CCALA 1120 under light microscope, after staining with Lugol's iodine solution (A–G) and alive (H–K). (A) Adult cell starts the process of differentiation into zoospores, (B, C) zoospores inside the mother cell wall. Pairing of gametes: (D–E) anisogamy, (F) isogamy, (G) a rounded

zoospore with two flagella, (H) young cell, (I) a large adult cell with differentiated zoospores, outside this cell are small rounded aplano-spores, (J) a rare pear-shaped adult cell, (K) small rounded aplano-spores. Scale bar = 10 μm

Fig. 2 5.8S rDNA-ITS2 ribosomal RNA gene-based Bayesian phylogenetic tree showing the phylogenetic position of *Bracteacoccus bullatus* CCALA 1120. Posterior probabilities (0.95 or more) and bootstrap values from maximum likelihood (50 or more) are shown. Thick branches represent nodes receiving the highest posterior probability support (1.00). The newly obtained sequence is in bold. Accession numbers and strain codes are indicated after each species name

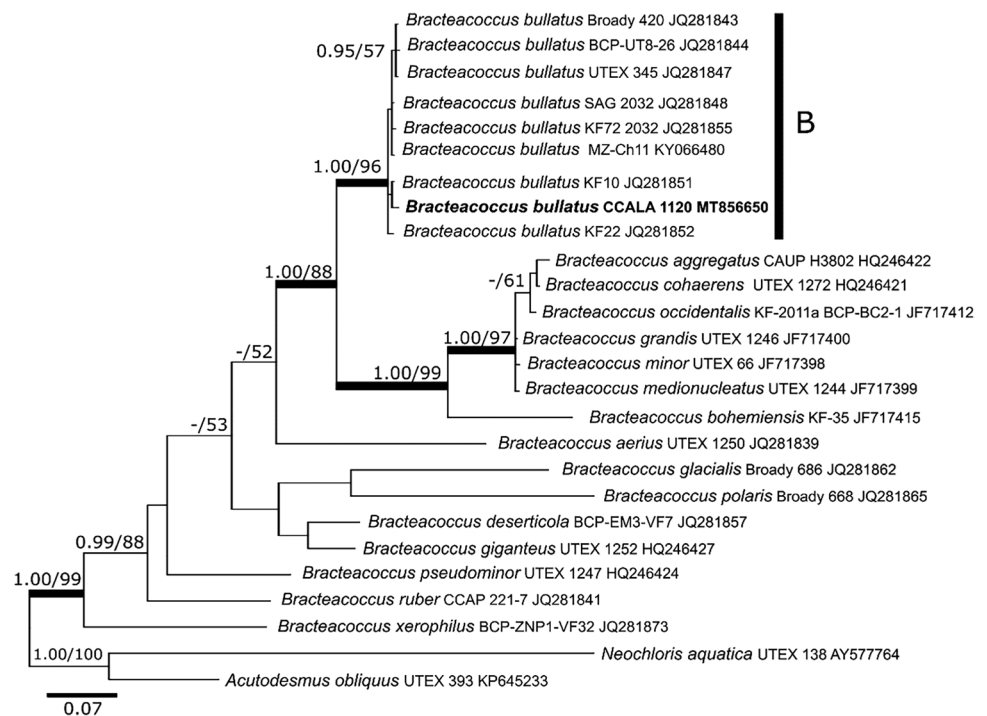
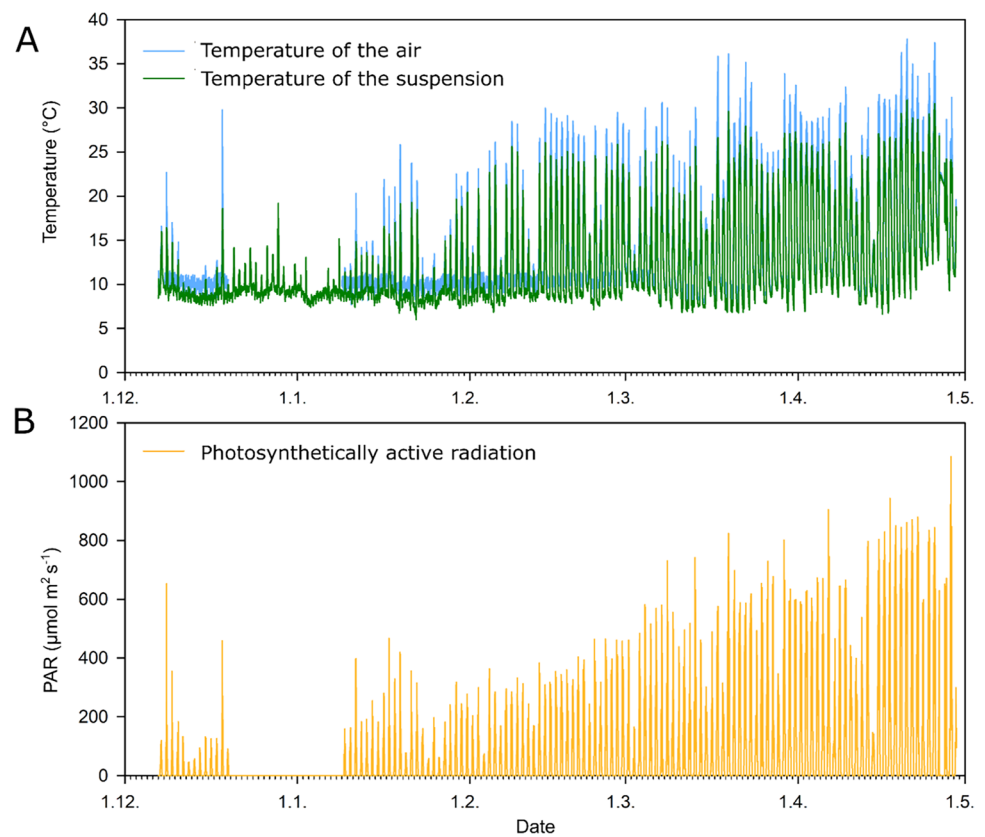


Fig. 3 Time course of (A) the temperature of the culture medium and air, (B) the PAR intensity in outdoor cultivation (greenhouse) on a platform. December 7 2018 – April 25 2019 (the third cultivation). Statistical summary of the data is included in Supplementary Table S2



gene, there was no sequence available for strain KF10 but the CCALA 1120 sequence was 99.9% identical with SAG 2032 (JQ259930; three bp differences out of 1783 bp). With the NCBI basic local alignment search tool server, the closest hit for ITS2 sequence of CCALA 1120 was *B. bullatus* strain KF10. It differed by two nucleotides bases. Therefore, the secondary structure of ITS2 of CCALA 1120 was predicted and compared with the *B. bullatus* type strain (SAG 2032); they differed by four nucleotides in single stranded regions of helixes I to III (Supplementary Fig. S2). Based on light microscopy of the cell morphology and the absence of CBC in the entire ITS2 structure, the CCALA 1120 strain was identified as *B. bullatus*. CCALA 1120, differing by four bp in ITS2 from MZ-Ch11 (Supplementary Fig. S2).

Culture growth and abiotic conditions

The temperature profiles of the medium and the air, and the intensity of PAR during the first, second and third cultivation periods are shown in Supplementary Fig. S3 and Fig. 3, respectively.

Temperature and light growth requirements

Cultivation in crossed gradients of temperature and light revealed a temperature optimum of about 21 °C (GAM,

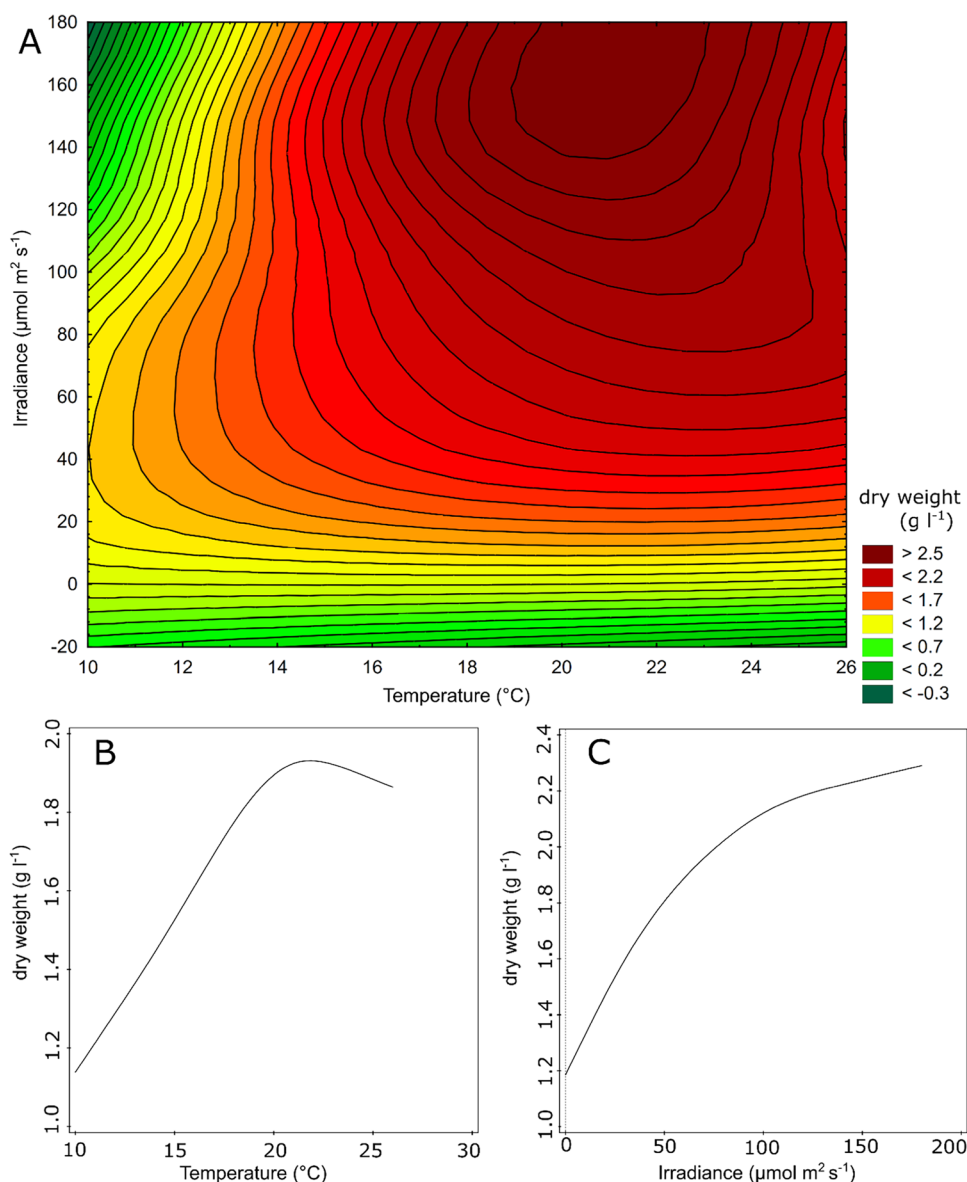
$n = 35$, $F = 3.5$, $P = 0.042$) and an optimum PAR of above 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (GAM, $n = 35$, $F = 12.7$, $P < 0.001$, Fig. 4). The maximum biomass yield was 2.88 g DW L^{-1} , corresponding to a mean productivity of 0.078 g DW $\text{L}^{-1} \text{day}^{-1}$. The effects of temperature (Monte Carlo permutation test, $n = 35$, pseudo- $F = 4.8$, $P = 0.05$, RDA: 12.7% of explained variation), effect of PAR (Monte Carlo permutation test, $n = 35$, pseudo- $F = 21.2$, $P = 0.002$, RDA: 39.1% of explained variation) and their combination (Monte Carlo permutation test, $n = 35$, 1st axis pseudo- $F = 32.1$, $P = 0.002$, all axes pseudo- $F = 16.0$, $P = 0.002$) on final biomass yield were found to be statistically significant.

Lipid and PUFA production

The strain was cultivated in three independent experiments, and the environmental conditions during individual cultivations are summarized in Supplementary Table S2. The third cultivation involved the complete cold period of the year.

The exponential phase of the growth curve proceeded for 20 days and growth was measured as Abs_{750} ; dry weight was determined after 40 days. Maximum yield was over 20 g of dry weight L^{-1} (Fig. 6). From the second cultivation, we harvested a total of 1271 g of dry weight (DW). The daily dry mass production rate was 2.72 g DW $\text{m}^{-2} \text{day}^{-1}$, and 0.22 g DW $\text{L}^{-1} \text{day}^{-1}$. The total content of fatty acids comprised

Fig. 4 Temperature and light requirements for growth of *Bracteacoccus bullatus* CCALA 1120. **(A)** Growth in crossed gradients of temperature and light. LED light type day. Biomass in dry weight, evaluated gravimetrically at the end of cultivation. **(B)** Generalized additive model of dependence of biomass yield on temperature **(C)** Generalized additive model of dependence of biomass yield on irradiance



unsaturated fatty acids at 53% of dry weight, and 18.3% LA, 17.4% ALA, 22.6% OL and 2.4% of vaccenic acid. Levels of the most prominent individual FAs are plotted in Table 1, total FAs reached a maximum after about 60 days of cultivation, LA about 80 days, ALA about 50 days, HDA about 30 days, and OL was growing the whole time, 110 h, these FAs are considered essential (Fig. 6).

Total pigment concentrations

HPLC peak spectra (Supplementary Fig. S4) showed that the dominant pigments were lutein ($R_t = 14.5$ min), chlorophyll *b* ($R_t = 18.77$ min), chlorophyll *a* ($R_t = 20.78$ min) and β -carotene ($R_t = 23.45$ min.). The minor pigments were as follows: neoxanthin ($R_t = 9.7$ min), violaxanthin ($R_t = 10.6$ min) and antheraxanthin ($R_t = 12.6$ min, Fig. 7). There were also

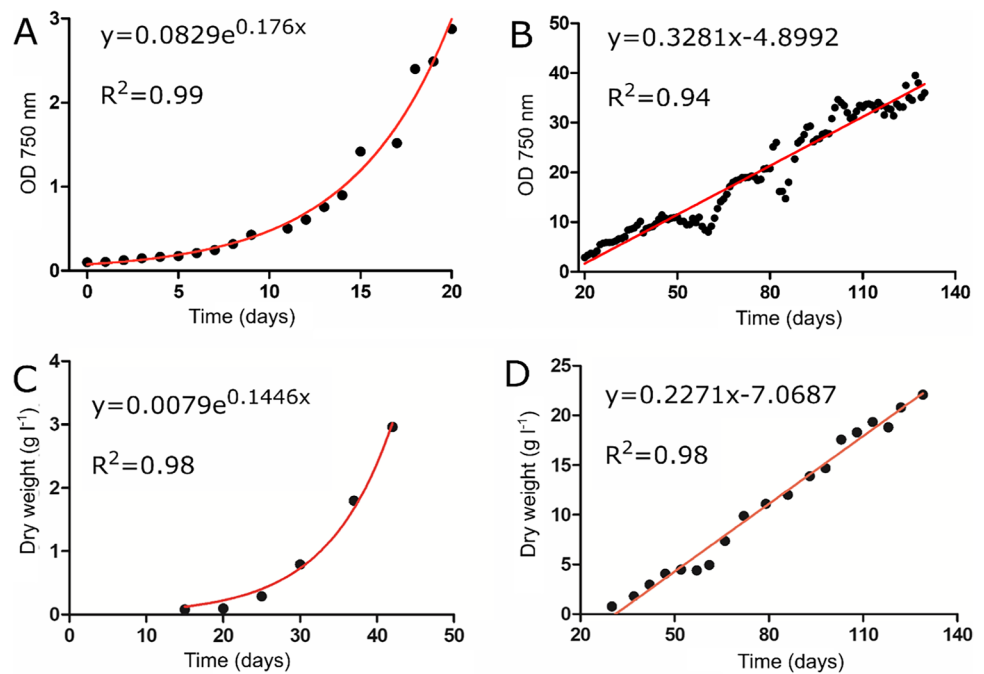
typical secondary carotenoids present in the chromatogram: echinenone (E) eluting at a retention time $R_t = 20.05$ min, astaxanthin monoesters (AXTme) eluting between retention times 21.58 min and 22.1 min, and astaxanthin diesters (AXTde) eluting on the end of the chromatogram between retention times 25 min and 28 min (Fig. 7, Supplementary Fig. S4). The carotenoid content was 10 mg L⁻¹.

Discussion

Identity and morphology

Bracteacoccus is a common terrestrial green algal genus that occurs in a wide range of soil types worldwide, spanning habitats from polar (e.g. Broady 1984; Stibal et al.

Fig. 5 Growth curves in pilot-plant cultivation of *Bracteacoccus bullatus* CCALA 1120. Fitting the data with the optimal equation using evaluation of growth as (A, B) absorbance at 750 nm (Abs_{750}), (C, D) dry weight. December 7 2018 – April 17 2019 (the third cultivation)



2006), alpine (e.g. Gärtner 1985), subtropical (Venter et al. 2018) to tropical (Neustupa and Škaloud 2008). Here, the investigated alga was isolated from mountainous snow in Spain. This alga appeared in further isolates from snow (J. Lukavský—pers. comm.). Several species in the genus *Bracteacoccus* were also reported from other extreme cold habitats including green ice in Antarctica (Kol and Flint 1968), glacial ice on Svalbard (Leya 2020), the coastal zone of the White Sea, Arctic (Chekanov et al. 2019) or tundra soils affected by coal mine pollutions (Patova and Dorokhova 2008). In Chlorophyceae, the ITS2 molecular

marker possesses enough resolution to distinguish between species (e.g. Matsuzaki et al. 2019; Trentin et al. 2022). Here, we showed that a unicellular alga CCALA 1120 is conspecific with several *B. bullatus* strains including authentic strain SAG 2032 isolated elsewhere, e. g. from soil in mining dump, contaminated with heavy metal in Germany (SAG 2032), from soil in Etna in Italy (strain KF10; Fučíková et al. 2012), the *Robinia* forest in Ukraine (strain MZ-Ch11; Mamayeva et al. 2018) and sites in North America, Arctic and Antarctic (Fučíková et al. 2012). Most recently, a new lineage of this species has recently been found in the semiarid zone in Chile (Samolov et al. 2020), indicating its widespread geographic distribution and ability of this species to colonize different habitats.

Reproductive cells are highly variable over time. Inside the mother cell, spindle-shaped zoospores, with two flagella and a stigma are first formed, vigorously rotating in one direction. If they manage to break free from the mother cell, they can mate as gametes, and anisogamy has also been observed. If they are not released they round up and forming autospores, these can later be released from the cell wall and grow up (Fig. 1).

As an addition to the monograph of Fučíková et al. (2012), this study documented sexual reproduction as the pairing of gametes and also an-isogamy in strain CCALA 1120 of *B. bullatus*. This offers opportunities for classic genetic experiments and breeding, and also that hybridization can take place during speciation within the genus. The production of zoospores and gametes was stimulated under a period of dark in the closely relative species, *B. minor* (Příbyl 2013).

Table 1 Content of fatty acids in total fatty acids produced during pilot-plant cultivation of the alga *Bracteacoccus bullatus* CCALA 1120, April 4 – June 9 2016 (the first cultivation)

Common name	Fatty acid	Abbreviation	[%]
Palmitic	16:0		15.6
Palmitoleic	16:1 ω -7	PA	3.1
	16:1 ω -9		1.2
7,10-velké Hexadecadienoic	16:2 ω -6		6.0
Hexadecatrienoic	16:3 ω -3	HDA	5.2
Hexadecatetraenoic	16:4 ω -3		5.1
Stearic	18:0		2.1
Oleic	18:1 ω -9	OL	22.6
	18:1 ω -7		2.4
Linoleic	18:2 ω -6		18.3
γ -Linolenic	18:3 ω -6	GLA	0.4
α -Linolenic	18:3 ω -3	ALA	17.4
Stearidonic	18:4 ω -3		0.6

Fig. 6 Time course of (A) total fatty acid content (%) in dry weight, (B) six selected fatty acid contents (% of total lipids) during the course of outdoor CCALA 1120 cultivation (greenhouse) on a platform, December 7 2018 – April 17 2019 (the third cultivation)

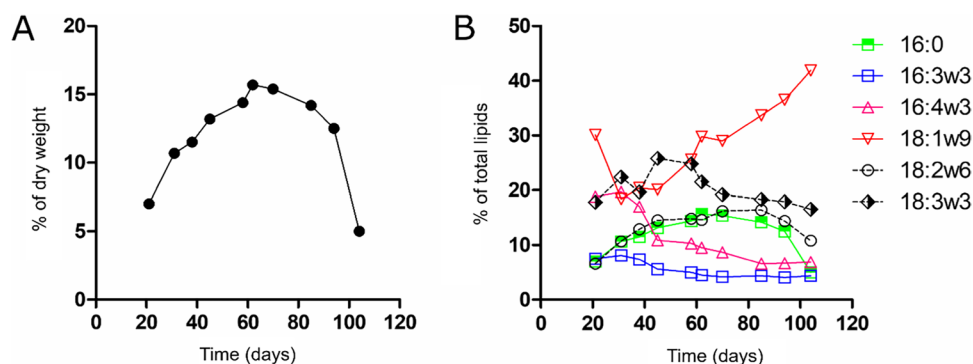
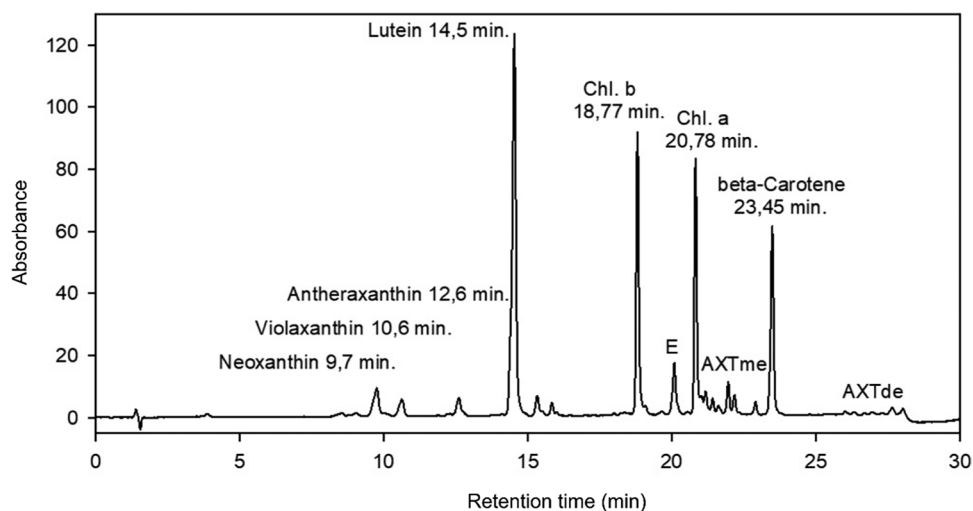


Fig. 7 HPLC analysis of *Bracteacoccus bullatus* CCALA 1120 at 450 nm (AXTme—astaxanthin monoesters, AXTde—astaxanthin diesters)



Growth optima, fatty acid and pigment composition and biotechnological potential

In general, strains with a broad temperature tolerance and the ability to grow at lower temperatures are prospective for mass production in winter, in temperate zones, or in summer in the polar regions (Kvíderová et al. 2017). In this study, the extremotolerant character of the *B. bullatus* strain is an advantage for biotechnology since it is possible to cultivate this strain during periods of lower temperatures and to extend the exploitation of cultivation units. Another strain, *B. aggregatus* had very similar optimal temperature and light values (21 °C, 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), as we obtained for our strain in crossed gradients of temperature and light, *B. aggregatus* was growing under 20 °C and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, when cultured it in a 600 mL bubble column on BG-11 medium (Chekanov et al. 2021). Difference in light intensity can be explain with bubbling of columns.

The growth optima of the experimental strain (temperature ca 21 °C, PAR above 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) were comparable to *Bracteacoccus* sp. N2 isolated from the soil in Ny Ålesund, Svalbard (Shukla et al. 2020). Unfortunately, Maltsev et al. (2020) did not mention the cultivation

temperature, but PAR used in growth experiment was slightly lower than our maximum, and lied within PAR optimum range after Shukla et al. (2020). Higher requirements for PAR were found in *Bracteacoccus pseudominor* BERC09 isolated from Pakistan (Malik et al. 2022).

Resistance to lower temperatures is obviously the result of the high levels of PUFAs (López et al. 2019). The strain is also sufficiently resistant to mechanical forces during pumping and against contamination by other algae; thus it can be grown in common cultivation units used for commercial production of e.g. *Arthrospira* (*Spirulina*) or *Chlorella* (Papapolymerou et al. 2018).

Strain *B. bullatus* CCALA 1120 contains PUFAs ω -6/ ω -3 in the ratio 1:1.16, meaning it is relatively close to the ideal ratio of 1:1, which is recommended by the World Health Organization. Manipulation of nutrient concentrations can also control the ratio of biomass to lipid production in order to increase the total lipid content from 17 to 59%, oleic FA to 48–64% and linoleic acid, to 14–24% (Mamaeva et al. 2018) which is comparable to the PUFAs content of our strain (53% in dry matter).

It is a common phenomenon in oleaginous green algae where, under normal conditions, they contain about 25%

w/w oil but under stress conditions, FAs can reach up to 45% w/w (Hu et al. 2008). This high content of FAs can be exploited in biodiesel production using *B. bullatus* (Mamaeva et al. 2018).

The values of FAs contents in our alga were obtained during long-term cultivation (over 100 h, Fig. 5 and 6) and without supplementing nutrients, i.e. under conditions of nutritional stress. Inducing cold or light stress is also possible, but it is much more expensive.

The total carotenoid yield of CCALA 1120 was 10 mg L⁻¹. Of the carotenoids detected in *Bracteococcus* alga (Fig. 7), the carotenoids neoxanthin, violaxanthin, antheraxanthin, lutein and beta-carotene participate in photosynthesis. The remaining carotenoids echinenone (E), astaxanthin monoesters (AXTme) and astaxanthin diesters (AXTde) generally do not participate in photosynthesis. These so-called secondary carotenoids serve primarily as protection of the photosynthetic apparatus against high light intensity. Further investigations should include an optimization of the cultivation unit for enhanced yield of carotenoids as an additional valuable biocompound formed under stress conditions and with aging of the culture (Minyuk et al. 2014). Prospective is also stimulation of carotenoids synthesis via UV_A irradiation (Chekanov et al. 2022).

Conclusions

The alga *B. bullatus* CCALA 1120 has been proven to be a good prospect for biotechnological cultivation. It produced a dry weight of 2.7 g m⁻² day⁻¹ i.e. 0.22 g L⁻¹ day⁻¹. With respect to content of PUFAs, linoleic acid and α -linolenic accounted for 18.3% and 17.4% of total fatty acids, respectively, and their production was 0.48 g m⁻² and 0.46 g m⁻² day⁻¹. The yield of total carotenoids was 10.11 mg.L⁻¹. PUFAs ω -6/ ω -3 were in the ratio 1:1.16, relatively close to the ideal ratio of 1:1. The alga is capable of being cultivated during cold periods, e.g. from December to April. This strain was patented (Lukavský et al. 2018).

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Author contribution J. Lukavský designed this study, isolated the strain, carried out the light microscopy, carried out the three large-scale cultivations and lyophilization processing. J. Kvíderová was responsible for statistical analysis and cultivation in crossed gradients, J. Kopecký performed the pigment analysis, D. Kubáč harvested the pilot-plant cultivation, L. Procházková sequenced the strain and performed the phylogenetic analysis and ITS2 rRNA secondary structure prediction, T. Řezanka carried out fatty acid methyl ester analysis. J. Lukavský, J. Kvíderová, L. Procházková and T. Řezanka wrote the manuscript, J. Kopecký, D. Kubáč, T. Řezanka contributed with their relevant parts. All authors discussed the results.

Data availability Data will be made available on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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