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

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Research Article

Keywords: Bracteacoccus bullatus, cryoseston, polyunsaturated fatty acids, carotenoids, pilot plant cultivation, biotechnology, molecular taxonomy.

Posted Date: October 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2125780/v1

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 2011/13) as being conspecific with *Bracteacoccus bullatus* (Chlorophyceae). For the first time this study documented sexual reproduction as the pairing of zoospores and also an-isogamy. Authentic strain SAG 2032. CCALA 1120 had a temperature optimum of about 21°C and an irradiance optimum above 160 µmol m^{-2} s^{-1}. It was cultivated in pilot-plant scale over three winter-to-spring periods from 2016 to 2019, using an open thin-layer photobioreactor in a greenhouse with only partial temperature control. After harvest, a high proportion of polyunsaturated fatty acids (PUFAs) was found (53% of dry mass) with linoleic (18:2ω-6) 18.3% and α-linolenic acids (18:3ω-3) 17.4% being the most abundant. Monounsaturated fatty acids accounted for about 30% with oleic (18:1ω-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 m^{-2} d^{-1} of dry weight, i.e. 0.2 g L^{-1} d^{-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.

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, 18:3ω-3) and LA (linoleic, 18:2ω-6) (Nakamura and Nara 2003). They also serve as precursors for substances biologically active in blood clotting, e.g. thromboxane or prostacyclin. Two groups of PUFAs are distinguishable, ω-3 and ω-6. The former are represented for instance by ALA and hexadecatrienoic acid (HDA; 16:3ω-3). The latter include, in particular, LA and γ-linolenic (GLA; 18:3ω-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; 18:1ω-9) occurs in various animal fats and vegetable oils. It is the precursor to the synthesis of essential 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).

Fatty acids are valuable products, but usually individual organisms predominantly produce only certain fatty acids. In addition, to cultivate an organism outdoors, it must be able to grow and be resistant to certain technological treatments during cultivation and harvest.

Algae, as a source of FAs, 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. 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. 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).

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)d *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 10-fold reduction in phosphorus and nitrogen in the nutrient solution resulted in OL, LA and palmitoleic acid (PA; 16:1ω-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% CO_2, under irradiance of 160 μmol m^{-2} 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), and these mainly absorb light at wavelengths from 400–550 nm (violet to green light for use in photosynthesis); they protect chlorophyll from photo-damage. Carotenoids that contain unsubstituted beta-ionone rings (including beta-carotene, alpha-carotene, beta-cryptoxanthin and gamma-carotene) have vitamin A activity and these and other carotenoids can also act as antioxidants. The carotenoid most frequently produced commercially is astaxanthin, whose industrial production is derived from plant- or animal-based and synthetic sources. The main algal source is *Haematococcus pluvialis* (Borowitzka 2013, Rajesh, et al. 2017). Astaxanthin is also found in yeast, salmon, trout, krill, shrimp, crayfish, crustaceans, and the feathers of some birds.

B. 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).

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 in 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 into 2L bottles of nutrient solution after Zachleder and Šetlík (1982), aerated by excess filtered (Millipore, Midisart 2000, PT FE IN, pores 0,2 µm) air with 2% CO_2 and irradiated by a white LED panel of light intensity 180 µmol m^{-2} sec^{-1} (although the first 2 days were supplied with only 90 µmol m^{-2} sec^{-1}). The bottles were placed in a water bath at a temperature of 7–10 ^{0}C. After pre-cultivation, the inoculum was transferred into a pilot-plant cultivation unit (BSC Engineering Brno, CZ), of volume 150 L, area 12 m^2, length 12 m, thickness of suspension 10–15 mm, located in a greenhouse (Doucha and Lívanský 1996, Supplementary Figure 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^{o}C. Nutrient solution (Zachleder and Šetlík 1982) was diluted to ½ with tap water, CO_2 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 QTi dataloggers (Minikin EMS, Brno, CZ). Growth curves were evaluated every day by absorbance at 750 nm in a spectrophotometer (Shimadzu UV-1800 PC) in 1 cm cuvettes (after dilution < 0.8 Abs_{750}). 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 x g, frozen to -20°C and lyophilized under 0.05 hPa in a 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 (Kvíderová 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 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 methylated 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 range of 50 to 600 Da. 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 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, and chlorophyll *a*, chlorophyll *b* and total carotenoid concentrations were calculated using the equations described by Lichtenthaler and Wellburn (1983): $Chl.a = 15.65*(A_{666}-A_{720})-7.34*(A_{653}-A_{720})$, $Chl.b = 27.05*(A_{653}-A_{720})-11.21*(A_{666}-A_{720})$, $Total\ carotenoids = 4.08*(A_{470}-A_{720})-0.0117*Ca-0.527*Cb$.

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, Deerfield, IL, USA). The extracts were further analyzed by high-performance liquid chromatography (HPLC) (Agilent Technologies Inc., Palo Alto, CA, 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) 100Å 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 (Lichtenthaller and 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 rDNA), internal transcribed spacer region 1 (ITS1 rDNA), 5.8S rDNA, internal transcribed spacer 2 (ITS2 rDNA) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) 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 the National Center for Biotechnology Information (NCBI) Nucleotide sequence database (18S + ITS1 + 5.8S + ITS2 rDNA: MT856650; *rbc*L: MT863786).

Secondary structure prediction of nuclear rDNA ITS2 and phylogenetic analyses

The methods of annotation and prediction of the secondary structure of the nuclear rDNA ITS2 region were the same as those described by Procházková et al. (2018). The concatenated 5.8S rDNA-ITS2 rDNA 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) and maximum likelihood (ML) 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 analyses and Bayesian posterior probabilities were performed as described by Nedbalová et al. (2017). 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 rDNA 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 rDNA 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 digital camera and an HI 100x/1.35 objective.

Statistical analyses

Statistical analyses were performed using Statistica 13.2 software (Dell 2013) and CANOCO 5 (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 aplanospores. (Fig. 1K). In old cells, a red pigment was produced.

Taxonomic identity of the strain using molecular 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 *rbc*L, the partial sequence (930 bp) obtained for this marker of CCALA 1120 was identical with the *B. bullatus* strain KF10 (JQ259875), consequently CCALA 1120 has the same phylogenetic position for this marker as strain KF10 (see *rbc*L phylogeny in Fig. 2 in Fučíková et al. 2012). Concerning 18S rDNA, 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). ITS2 rDNA is a non-coding region between 5.8S and 26S rDNA. This hypervariable molecular marker is 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). With the NCBI basic local alignment search tool server, the closest hit for ITS2 sequence of CCALA 1120 was *B.bullatus* strain KF10 (Fučíková et al. 2012). It differed

by two nucleotides bases. Therefore, the secondary structure of ITS2 rDNA 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 Figure S2). Based on light microscopy of the cell morphology and the absence of CBC in the entire ITS2 rDNA structure, the CCALA 1120 strain was identified as *B. bullatus*. CCALA 1120, differing by four bp in ITS2 rDNA from MZ-Ch11 (Mamaeva et al. 2018) (Supplementary Figure 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 Figure 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 ^{o}C (GAM, n = 35, F = 3.5, P = 0.042) and an optimum PAR of above 140 µmol m^{-2} 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 L^{-1} d^{-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 m^{-2} day^{-1}, and 0.22 g DW. L^{-1} day^{-1}. The total content of fatty acids comprised 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).

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	16:1ω-7	PA	3.1
	16:1ω-9		1.2
7,10-hexadecadienoic	16:2ω-6		6.0
Hexadecatrienoic	16:3ω-3	HDA	5.2
Hexadecatetraenoic	16:4ω-3		5.1
Stearic	18:0		2.1
Oleic	18:1ω-9	OL	22.6
Cis-vaccenic	18:1ω-7		2.4
Linoleic	18:2ω-6		18.3
γ- Linolenic	18:3ω-6	GLA	0.4
α- Linolenic	18:3ω-3	ALA	17.4
Stearidonic	18:4ω-3		0.6

Total pigment concentrations

HPLC peak spectra (Supplementary Figure 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, Supplementary Figure S5). 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 (Supplementary Figure S5). 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. 2006), alpine (e.g. Gärtner 1985),

subtropical (Venter et al. 2018) to tropical (Neustupa & 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 & 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 & Dorokhova 2008). In Chlorophyceae, the ITS2 rDNA molecular marker possesses enough resolution to distinguish between species. Here, we showed that a unicellular alga CCALA 1120 is conspecific with several *B. bullatus* strains isolated elsewhere, e. g. from soil in Etna in Italy (Fučíková et al. 2012) or the *Robinia* forest in Ukraine (Mamayeva et al. 2018). 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.

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

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 prospects 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.

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). 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 total carotenoid yield of CCALA 1120 was half that of *Haematococcus pluvialis* cultivated under blue LED illumination (0.25 μg mL^{-1}; Katsuda et al. 2014). 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 (e.g. Minyuk et al. 2014).

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).

Abbreviations

ALA = α-linolenic acid, FAs = fatty acids, GAM = generalized additive models, GLA = γ-linolenic acid, HDA = hexadecatrienoic acid, LA = linolenic acid, OL = oleic acid, PA = palmitoleic acid, PAR = photosynthetically active radiation (400 – 700 nm), PUFAs = polyunsaturated fatty acids, MUFAs = monounsaturated fatty acids, RDA = redundancy analysis.

Declarations

Funding statement

This research was funded by The Technology Agency of the Czech Republic (projects TE 01020080, and TN 0100048), Inst. Botany of the Czech Acad. Sci. (AVOZ60050516), the Ministry of the Education Youth and Sports (LTAIN19139) and by the institutional long-term research plan of the Inst. Botany of Czech Acad. Sci. (RVO 67985939 and AVOZ60050516), Charles University Res. Centre program 204069 and Inst. Microbiology, Czech Acad. Sci. RVO61388971, and European Union's Horizon 2020 research and innovation programme under grant agreement No 887227.

Acknowledgements

We thank doc. V. Cepák for collecting samples, H. Brabcová, ing. S. Pumpr and J. Hinterholzinger for technical support, last, but not least Prof. J.D.Brooker for language dressing the manuscript.

Author contribution statements

J. Lukavský designed this study, isolated the strain, carried out the light microscopy, carried out the three large-scale cultivations and lyophylization 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.

Competing interests

The authors declare no competing interests

Data availability

Data will be made available on reasonable request.

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Figures

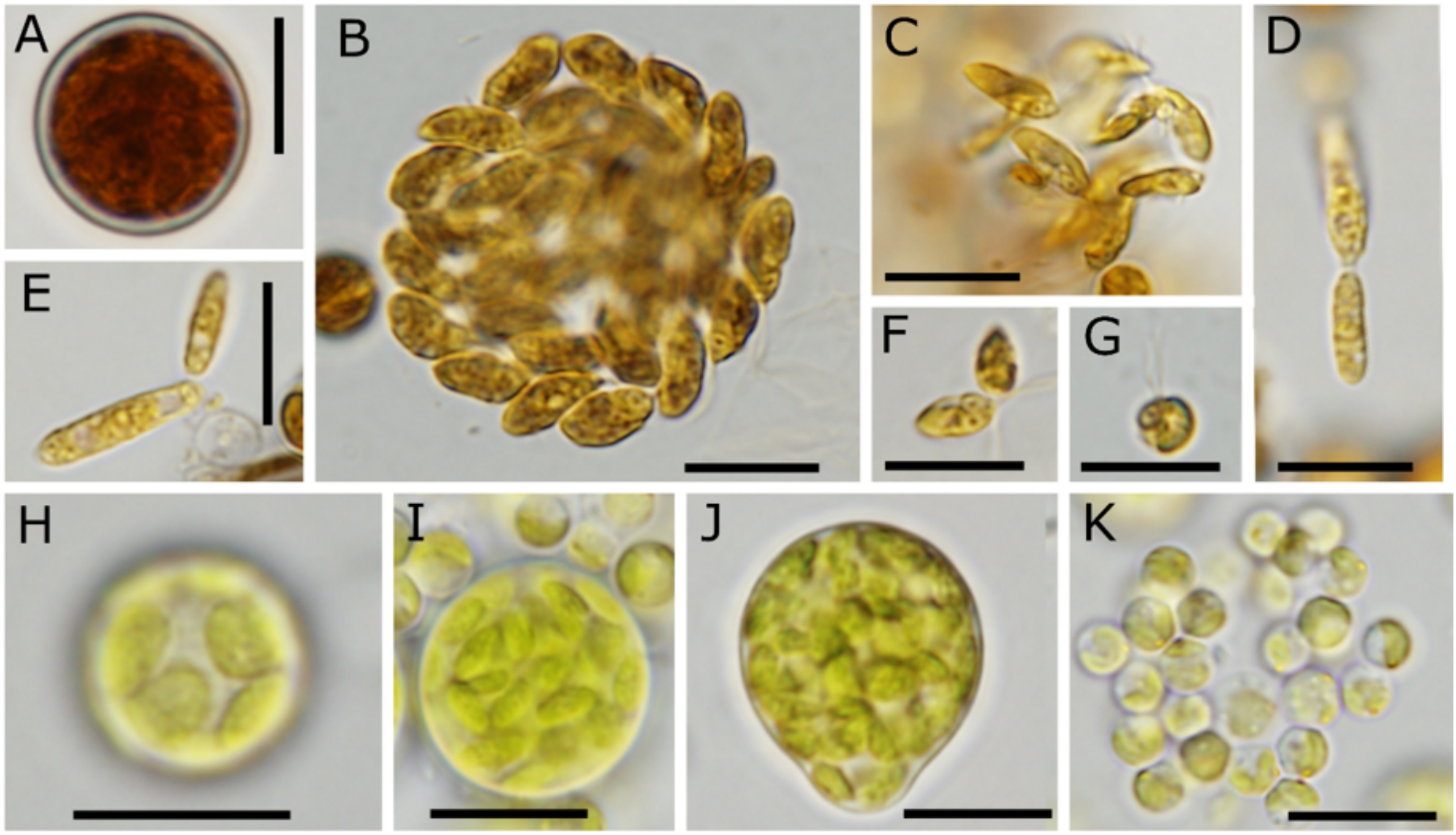

Figure 1

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 aplanospores, (J) a rare pear-shaped adult cell, (K) small rounded aplanospores. Scale bar = 10 µm.

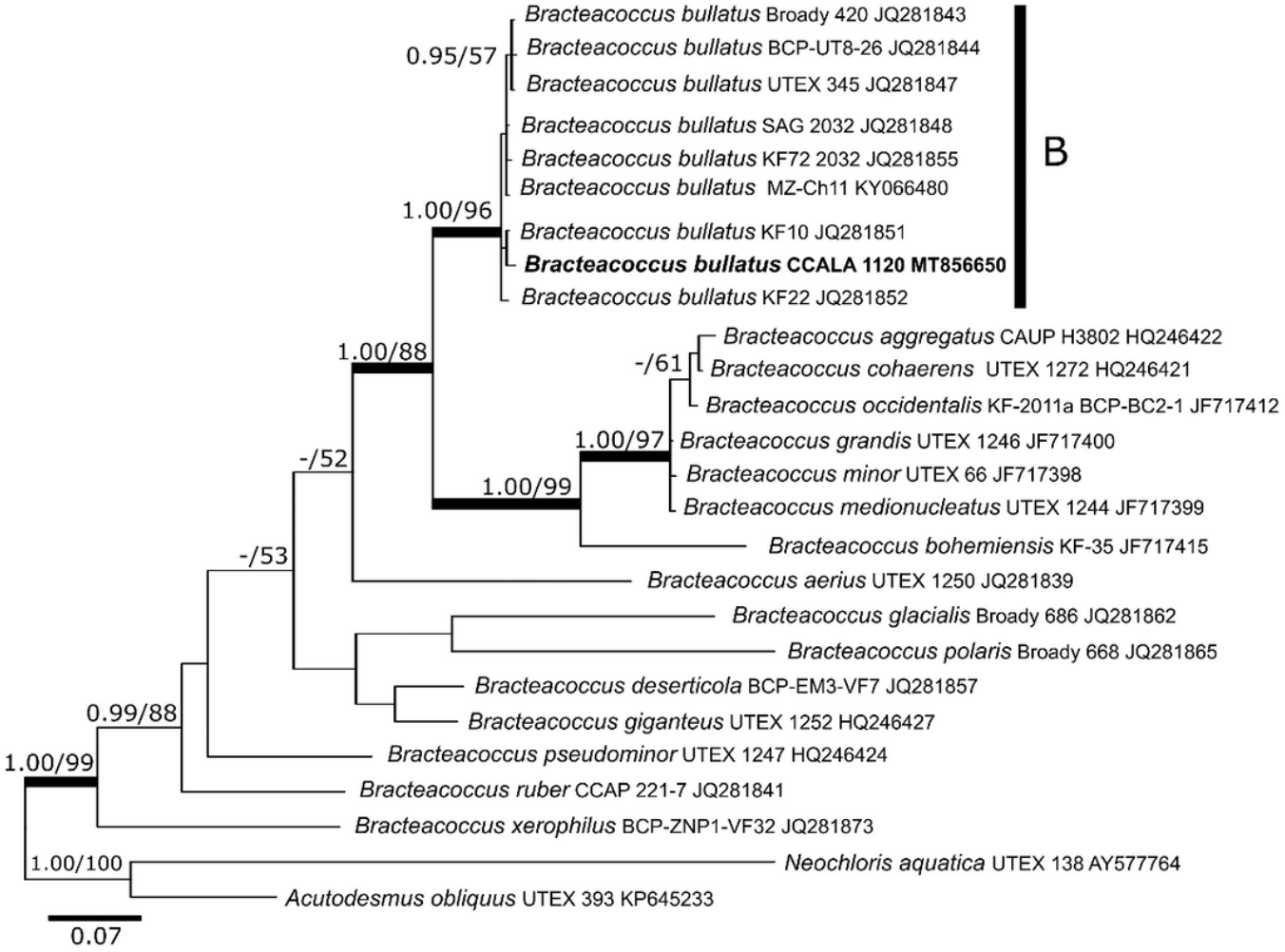

Figure 2

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.

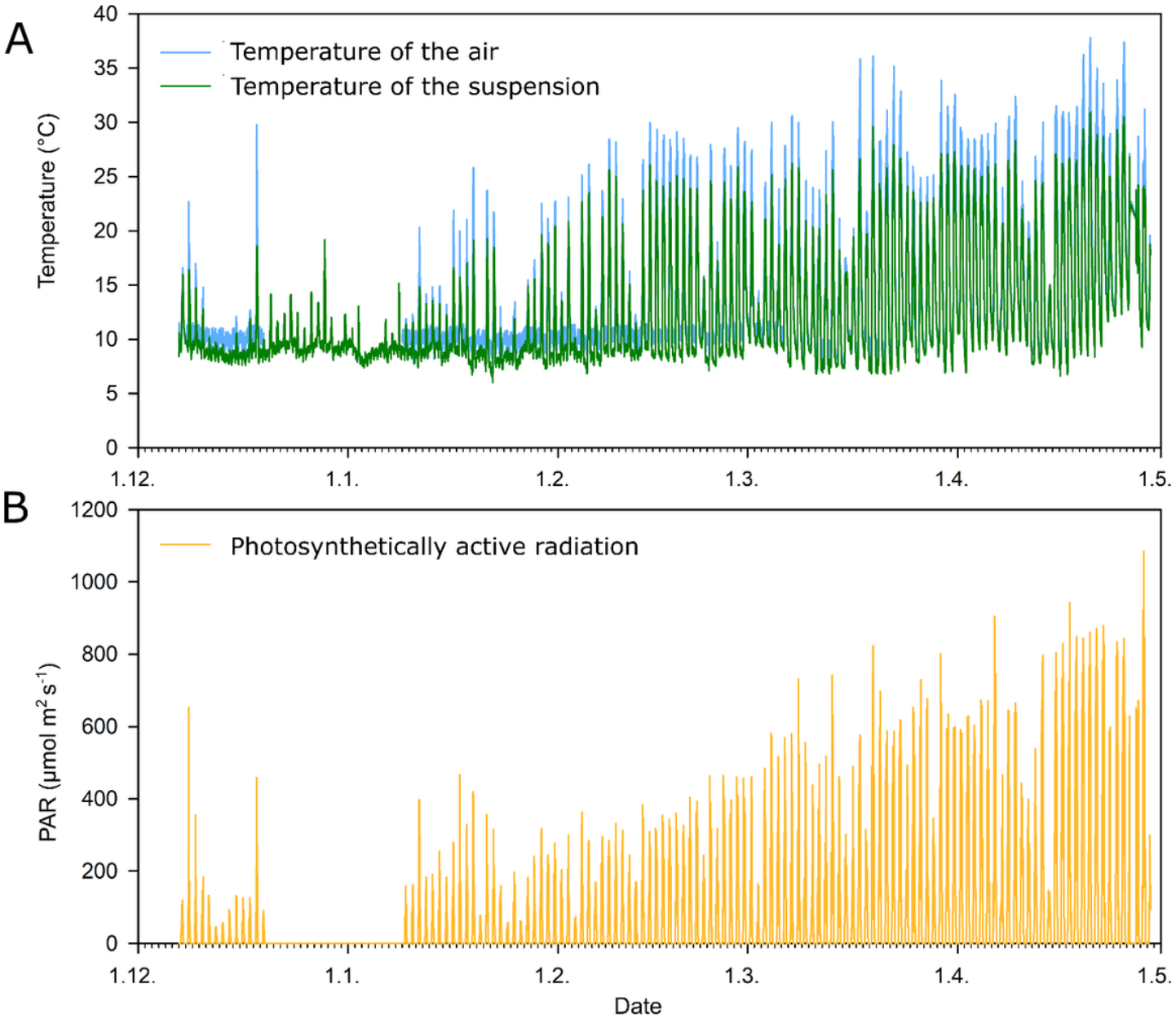

Figure 3

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.

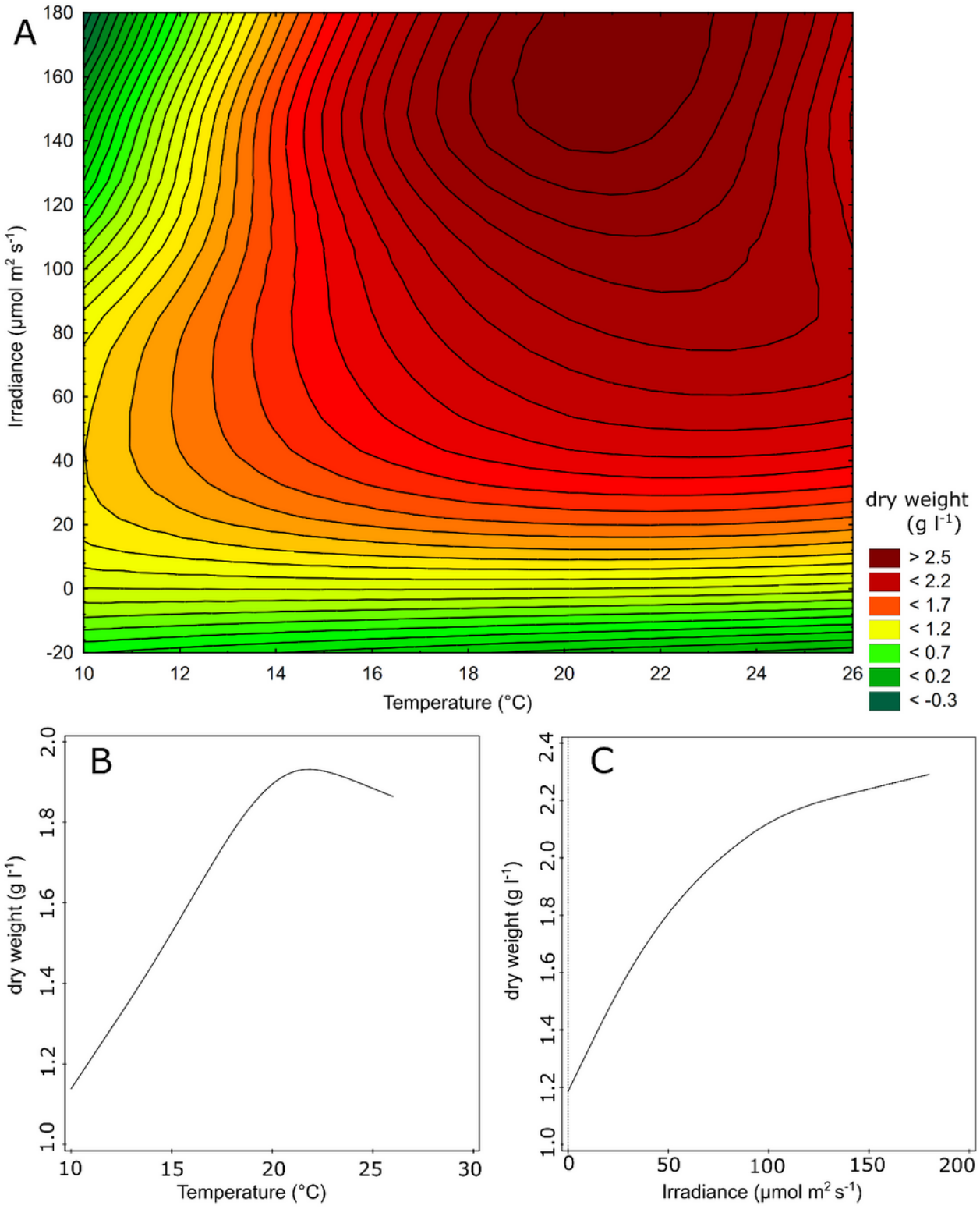

Figure 4

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.

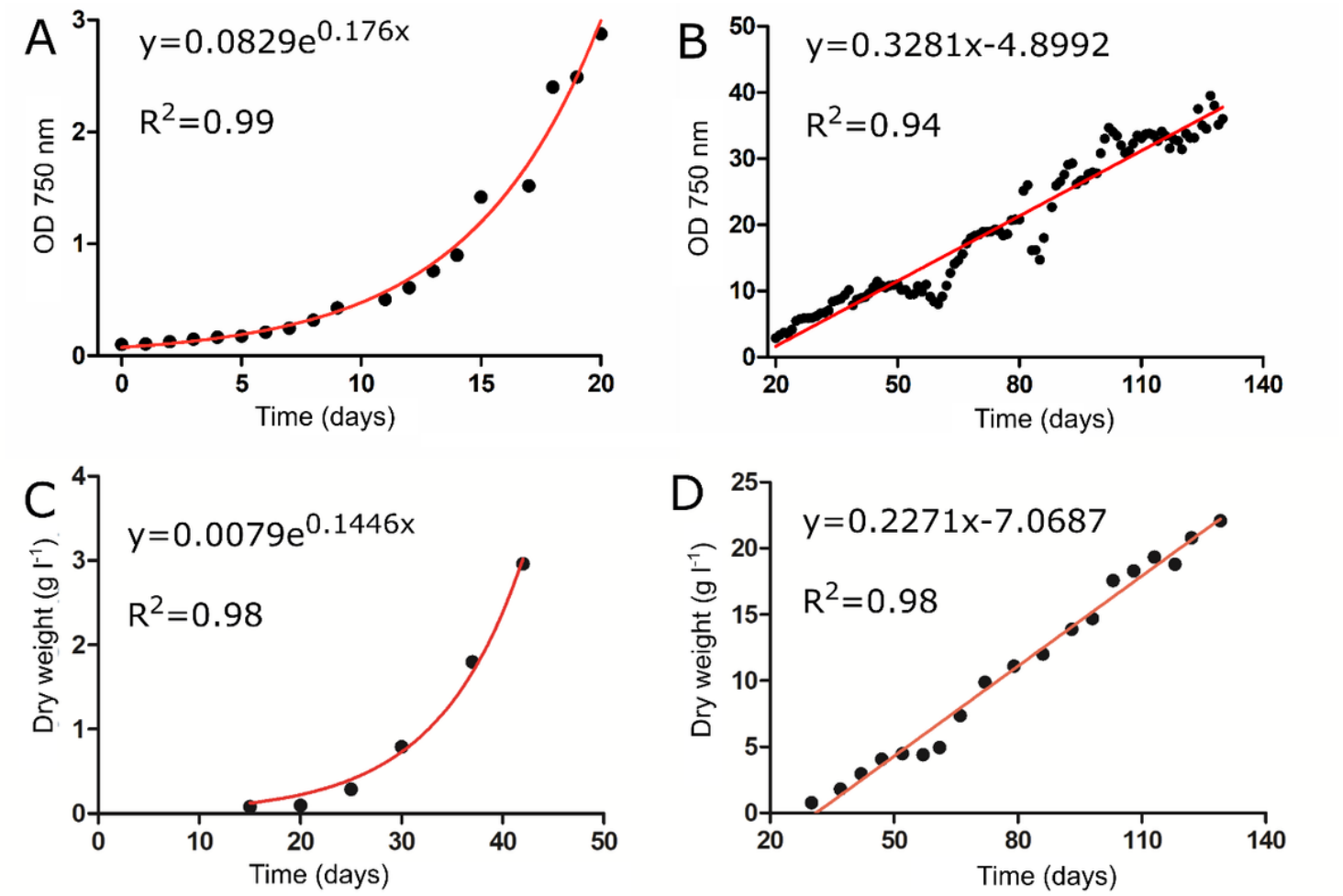

Figure 5

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).

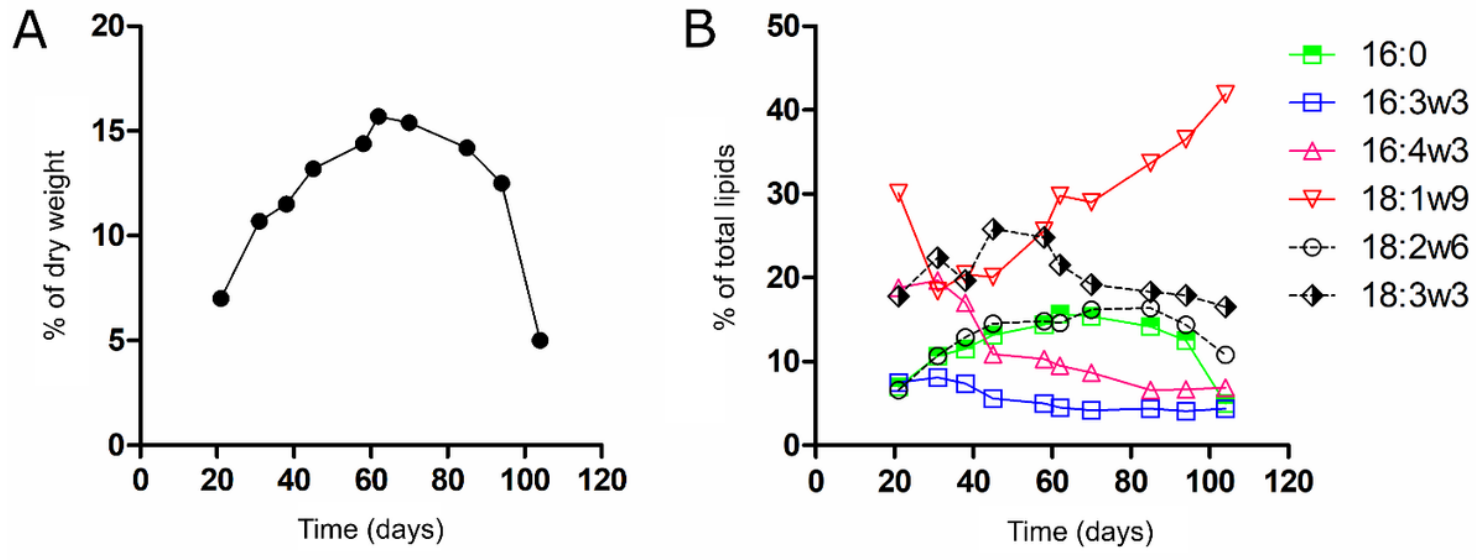

Figure 6

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).

Supplementary Files

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