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Simultaneous production of γ-linolenic acid and carotenoids by a novel microalgal strain isolated from the underexplored habitat of intermittent streams

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ABSTRACT

This study provides an in-depth investigation of biotechnological potential of a novel strain of microalga *Pseudomuriella engadinensis* KASU1, isolated from intermittent stream in the temperate region of Central Europe. Survival in drying environment of the intermittent streams requires strategies that are often related to the production of unique compounds. The newly isolated strain grows fast (doubling time as fast as 12 h) and robustly under a range of nitrogen concentrations (1.76–17.6 mM), light intensities (100–1200 μE m^{-2} s^{-1}) and temperatures (20–25 °C), as well as under CO_2 levels as high as present in the flue gas (up to 10% CO_2). *P. engadinensis* produces essential fatty acids such as oleic acid, linoleic acid and γ-linolenic acid, and carotenoids such as β-carotene and lutein. γ-Linolenic acid was produced with the highest rate reported for microalgae (44 mg L^{-1} d^{-1}), and lutein was produced with significantly higher rate compared to marigold petals traditionally used for its production. *P. engadinensis* showed high potential for nutraceutical production and waste gases treatment since it fixed up to 83 mg carbon L^{-1} d^{-1}.

1. Introduction

The challenges for the 21st century, including mitigation of climate change, food and fresh water supply security, or sustainable energy development make the use of microalgae as a human resource of high importance [1]. Though microalgae have gained a lot of attention as a valuable source of proteins, carbohydrates, fatty acids (FA), carotenoids, or vitamins [2], only a few species are currently used for commercial production of these nutraceuticals, including *Arthospira* spp. (*Spirulina* – a commercial name), *Chlorella* spp., *Dunaliella salina* and *Haematococcus pluvialis* [3]. A number of works concerned the isolation of microalgal species from natural localities or from wastewaters, and demonstrated potential of the isolated strains for the production of valuable compounds [4–6]. Microalgae are often isolated from extreme environments where the survival depends on the synthesis of efficient antioxidants, and where membrane fluidity is maintained by the synthesis of unsaturated FA [7]. For instance, López et al. isolated a strain of thermoacidophilic red microalga *Galdieria* sp. from an acidic hot spring in Colombia, and detected long-chain polyunsaturated fatty acids (PUFAs) in its FA profile [8]. Treves et al. focused on the isolation of microorganisms from a desert sand crust in Israel, which is a truly extreme site in terms of light intensities and temperature amplitude [9]. A strain of *Chlorella ohadii* isolated in their study exhibited structural changes that allowed for extraordinary efficient photosynthesis, metabolism and growth. These and other findings (for review, see [10]) underpin the importance of searching for novel organisms in natural habitats. Despite the variety of habitats studied for microalgae isolation, the intermittent streams in the continental temperate regions has until now remained completely unexplored for microalgae physiology, in contrast to Mediterranean climates. Catchment belonging to continental, humid climate

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[11] are characterized by hot summers where the air temperature exceeds frequently 35 °C. The maximum measured streambed surface temperature at the site selected for microalgae isolation in this study, described previously [12], was 56.6 °C.

Intermittent streams, known also as temporary or ephemeral, are characterized by the repeated occurrence of a dry period during which they cease to flow, or when the water completely disappears from the riverbed surface [13]. The sudden stream intermittence exposes the less adapted temperate stream communities to strong pressures of harsh environmental factors. Only a limited pool of taxa can thrive in such extreme conditions, characterized by pronounced seasonal (frequent switch between flow and dry phases) and also diurnal oscillation (such as rapid changes in the riverbed temperature in streams with reduced riparian vegetation [14]). The adaptive traits include accumulation of protective pigments, energy storage compounds, or desiccation protectants [15]. The current study is focused on an in-depth investigation of the production of FA and carotenoids by microalga *Pseudomuriella engadinensis* KASU1 isolated from an intermittent stream in the temperate region of Central Europe. *P. engadinensis* is a little known species, its biotechnological potential has been evaluated only within a single previous study, on a strain isolated from soil of an artificial pine and robinia plantation in the steppe zone of Ukraine [16]. The Ukrainian strain contained relatively high concentrations of ω-3-PUFA important for nutrition, which indicated potential for its nutraceutical usage. However, a comprehensive study of accumulation of FA and other valuable compounds under a variety of cultivation conditions has not been addressed up to date. Specific goals of the present study were: (i) to evaluate growth rate and biomass productivity of *P. engadinensis* KASU1 under a range of light intensities, temperatures, and carbon and nitrogen availabilities, (ii) to assess variability in accumulation rates of FA and carotenoids, and (iii) to identify specific cultivation parameters that allowed for maximal yields and productivities, in order to evaluate potential of *P. engadinensis* KASU1 for biotechnological applications.

Of special interest was the accumulation of ω-3 PUFA, β-carotene and lutein, as nutrients beneficial for human diet. Especially the lutein production by microalgae is growing in importance, since typical lutein content in microalgae (0.5–1.2 % w/w) is significantly higher than content in marigold petals (0.03 % w/w) traditionally used for its production [17,18]. The selected abiotic factors (light intensity, temperature, N content) were shown previously to affect biomass, FA and carotenoids accumulation [8,18–22]. In addition, cultivation under increased CO_2 levels (up to 10% v/v) was tested, in order to assess cost-effective and sustainable microalgae production with the use of CO_2-rich industrial waste gases [23–25]. The current study shows the potential of intermittent streams as a pool of new species for isolation and investigation for the biotechnological use, especially for the production of the high-value nutraceutical substances.

2. Materials and methods

2.1. Sampling site characteristics

Biofilm samples were collected from the Jarkovec stream (48.8586019 N, 17.4035092 E, altitude 301 m a.s.l. [12]). Jarkovec is an intermittent stream located in the Czech Republic downstream Natural reserve Čertoryje in the White Carpathian Landscape protected area. The catchment of the stream is small (7.3 km^2, length 5.0 km, land use dominated by forest 73 % and meadows 27 %, free of any settlement) and the stream water stops flowing between spring and summer (June), when the stream channel can dry up completely (typically until November). The riparian forest is mainly composed of alder (*Alnus glutinosa*) and maples (*Acer platanoides*, *A. pseudoplatanus*, *A. campestre*) partially open tree canopies that do not sufficiently shade the riverbed to prevent surface from reaching high temperature in summers. Further details on the sampling site, precipitation and temperature profiles in the year of biofilm sampling 2019 are provided in Supplementary Fig. S1. Detailed characteristics of the Jarkovec stream are available at Pařil et al., 2019 [12] (Supplementary Material – Table S1).

2.2. Sample collection and algal strain isolation

Benthic biofilm was collected from the Jarkovec riverbed on July 2019 when the streambed was completely dry, mainly due to the reduced precipitation in months preceding the sampling (Supplementary Fig. S1). At the site, three samples of the mixture of small pebbles, sand and clay were collected by metal spoon. Only 5 cm of the upper streambed layer was sampled (no sampling cores were used). In total, 3 × 10 mL of the dry riverbed mixture was transferred into 3 × 50 mL plastic sampling tubes and flooded with 3 × 30 mL of BG-11cultivation medium [26]. In laboratory, the flooded samples were transferred to 3 × 250 mL Erlenmeyer flasks and flooded with liquid BG-11 medium to the total volume of 100 mL. The flasks were let to sit on a cultivation shelf under continuous illumination of 70 μE m^{-2} s^{-1} (cool white LEDs), ambient CO_2 atmosphere and a temperature of 25 °C. No shaking was involved and the flasks were observed daily for the appearance of green aggregates on the surface of pebbles, sand and clay, as a result of phototrophic growth of naturally present algal population.

The first green colonies were transferred by an inoculation loop onto solid agar BG-11 plates (2 % w/w) and incubated under the same conditions as the Erlenmeyer flasks. The colonies growing on agar plates were repeatedly examined by optical microscope (Olympus CX 31), and the exclusive presence of unicellular green microalgae was confirmed. After three cycles of colonies reinoculation onto fresh agar plates (each cultivation lasted for one week), the colonies were transferred again into liquid BG-11 medium in Erlenmeyer flasks, and the flasks were placed inside a cultivation chamber on a shaker (100 rpm) under continuous illumination of 70 μE m^{-2} s^{-1} (cool white LEDs), 2 % (20,000 ppm) CO_2 atmosphere and at 25 °C. The algal suspensions formed homogeneous culture of unicellular green microalgae, which was confirmed again by optical light microscopy as well as by 18S rRNA gene sequencing (see the next section for details). The stock cultures were further kept on the shaker (under ambient CO_2 atmosphere) and reinoculated into fresh BG-11 medium every 2–6 weeks.

2.3. DNA sequencing and phylogenetic analysis

Total genomic DNA was isolated from fresh algal biomass using NucleoSpin Soil kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. Prior to the extraction procedure, two parallel subsamples of the culture were centrifuged in 1.5 mL Eppendorf tubes using a benchtop minicentrifuge. The resulting pellet (approximately 100 mg wet biomass) was homogenized in buffer SL1 from the NucleoSpin Soil kit using 2 mm wolfram carbide beads and the Retsch MM200 ball mill (Retsch, Haan, Germany) at 30 s^{-1} frequency before starting the recommended protocol. PCR of the small subunit (18S) rRNA gene was achieved using previously published primer pairs 18SF/18SR [27] and NS1F/1650R [28] under the following cycling conditions: initial denaturation for 5 min at 94 °C; 38 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 50 °C, and elongation for 3 min at 72 °C; and a final elongation step for 10 min at 72 °C. The PCR products were sequenced commercially by the Sanger method at SeqMe company (Dobříš, Czech Republic) on both strands using the same primers as for the PCR amplification. Obtained raw sequencing data were assembled and quality-checked using Geneious Prime v. 2020.1.2 software package (Biomatters, Auckland, New Zealand) and the resulting consensus 18S rRNA gene sequence was submitted to GenBank under the accession number OM909161.

For phylogenetic analysis, the sequence of *Pseudomuriella engadinensis* KASU1 was aligned together with its closest BLAST hits and representative chlorophyte sequences according to relevant literature [29] using MAFFT v. 7 [30]; in total, the alignment included 27 sequences and 1611 aligned positions. A Bayesian Inference tree was

calculated with MrBayes v 3.2.6 [31] applying the GTR + I + G substitution model (lset nst = 6 rates = invgamma) using two parallel runs of eight heated chains for 1.1 million generations, sub-sampling frequency of 100 and trees sampled in the first 100 thousand generations discarded as burn-in. The resulting average standard deviation of split frequencies was lower than 0.01 at the end of the analysis, and the potential scale reduction factor of all parameters was between 0.99 and 1.00. A majority consensus Byesian tree was constructed, and the branch support was calculated as the estimated posterior probabilities of nodes based on their frequencies in the sampled trees. In parallel, a Maximum Likelihood tree was retrieved using the GTR + I + G substitution model and 1000 rapid bootstrap replications in RaxML v 8.0 [32].

2.4. Experimental setup

2.4.1. Algae cultivation

To characterize growth, productivity and biochemical composition of *P. engadinensis* KASU1 under a variety of cultivation conditions, the microalga was cultivated in Multi-cultivator MC 1000–OD (Photon Systems Instruments, Czech Republic). MC 1000–OD contained eight independent cultivation tubes (diameter 27 mm, volume 80 mL) immersed in a temperature-controlled water bath. Each tube was independently illuminated by cool white LEDs. MC 1000–OD was connected to a high-precision gas mixing system GMS-150 (Photon Systems Instruments, Czech Republic) which allowed to regulate the CO_2 concentration in the input gas between 450 and 10,000 ppm. Each cultivation tube was bubbled with a flow rate of 115 mL min^{-1}.

2.4.2. Experimental design

Structural and functional responses of *P. engadinensis* KASU1 to a variety of cultivation conditions was tested in three consecutive sets of experiments. The initial growth characterization was performed in full BG-11 medium under CO_2 concentrations of 450–4000 ppm, light intensities of 200–600 μE m^{-2} s^{-1}, and at 25 °C (Supplementary Table S1). Based on the results, the FA and carotenoids profiles were further characterized under a range of N concentrations in the BG-11 medium (1.76–17.6 mM), light intensities (100–600 μE m^{-2} s^{-1}) and temperatures (15–25 °C). The full set of cultivation conditions in all experiments is described in detail in Supplementary Table S1. In addition, the ability of *P. engadinensis* to grow at CO_2 concentrations up to 10,000 ppm was tested in full BG-11, at 200 μE m^{-2} s^{-1}, and at 25 °C (Supplementary Table S1). Each cultivation was performed in a batch regime and lasted up to seven days. This period was long enough to reach high culture densities and to allow the cells to accumulate high content of both FA and carotenoids within linear / stationary growth phase (Figs. 3-5). The initial cell density was set to approximately 200 mg_{DW} L^{-1}.

2.5. Analytical procedures

2.5.1. Growth rate and biomass productivity

Algal growth was monitored by MC 1000-OD using its automated measurement of optical density at both 680 nm (OD_{680}) and 720 nm (OD_{720}). The built-in OD sensor measures linearly only up to OD_{720} < 0.4 due to the light scattering by the cellular matter. The scattering increases exponentially with the cell density (see Supplementary Fig. S2 for details). To reconstruct the growth curves from the recorded OD_{720} data, $OD_{720} > 0.4$ was corrected by the following exponential regression model (the equation was obtained using least-square data interpolation method; $R^2 = 1.00$):

$$OD_{720\ corrected} = 0.029 + 0.143\ e^{2.497\ OD_{720\ measured}} \quad (1)$$

The OD_{720} correction allowed for the evaluation of specific growth rates (μ; unit $time^{-1}$) in the exponential growth phase according to the classical exponential regression model:

$$\mu = \frac{ln\frac{OD_{720\ t_2}}{OD_{720_{t_1}}}}{t_2 - t_1}, \quad (2)$$

where $OD_{720\ t1}$ and $OD_{720\ t2}$ represent OD_{720} corrected by Eq. (1), measured in times t_1 and t_2, respectively.

After three to seven days of each batch cultivation (see Supplementary Table S1), samples for cellular dry weight (DW) analysis were withdrawn form MC-1000-OD tubes. DW was measured gravimetrically using XA105DR analytical balances (Mettler Tolledo, Switzerland). This allowed to evaluate the culture productivity (g_{DW} L^{-1} d^{-1}).

To analyze the effect of temperature on *P. engadinensis* KASU1 growth, a temperature coefficient Q_{10} (unitless; a shift in the followed parameter with 10 °C) was calculated as:

$$Q_{10} = \left(\frac{(\mu \parallel P)_2}{(\mu \parallel P)_1}\right)^{\frac{10°C}{T_2 - T_1}}, \quad (3)$$

where $(\mu \parallel P)_1$ and $(\mu \parallel P)_2$ are either the specific growth rates (μ) or productivities (P) at temperatures T_1 and T_2, respectively.

2.5.2. Fatty acids and carotenoids analysis

Samples for both FA and carotenoids determination were withdrawn from the MC 1000-OD at the seventh day of the batch cultivation experiments, centrifuged (4500 ×*g*, 5 min, 21 °C), freeze-dried overnight (Cool Safe Basic, Trigon Plus, Czech Republic) and stored at −80 °C until further analysis.

Total carotenoid and chlorophyll content were determined using the HPLC/PDA method. Samples of freeze-dried algae biomass were mixed, weighed and rehydrated with 1 mL of MiliQ water. Excess water was removed by centrifugation and 1 mL of methanol and about 0.5 mL of glass beads (0.2–0.5 mm diameter) was added to each sample. The samples were vortexed for 20 min, transferred to 15 mL tubes and washed with 2 mL of chloroform. The mixtures were vortexed for 10 min, flooded with 1 mL of water, and the tubes were allowed to stabilize for two phases after shaking. The lower chloroform phases were quantitatively transferred to clean tubes and dried under an inert nitrogen atmosphere. The dried samples were dissolved in 1 mL of Ethyl Acetate: Acetonitil mixture (2:1) and filtered through a 0.45 μm PTFE filter into the glass vials. Samples were measured on Dionex Ultimate series HPLC with Vanquish DAD detector (Thermo Fischer Scientific, USA) on Kinetex C18-EVO column 150 mm × 4.6 mm × 5 μm (Phenomenex, USA) using gradient separation with mobile phase A (ACN:MeOH:Tris HCl pH = 8; 84:2:14) and mobile phase B (MeOH:EtAc; 60:40) at flowrate 1.2 mL min^{-1} and 25 °C. Carotenoids were detected at 445 nm and chlorophylls at both 445 nm and 455 nm. Chromatographic data were evaluated using Chromeleon 7.2. software. The pigment production was evaluated using commercial standards (Betacarotene, Lycopene, Neoxanthin, Violaxanthin, Astaxanthin, Lutein, Chlorophyll A and Chlorophyll B; all standards purchased from Sigma Aldrich, USA) and external calibration.

Total lipids and individual FA were determined by GC/FID analysis. Approx. 10–15 mg of freeze-dried algae biomass was put into 2.0 mL crimp neck vials together with 1.8 mL of 15 % (v/v) H_2SO_4 in methanol, capped with aluminum cap and heated at 85 °C for 2 h. After the transesterification process, the mixtures were transferred quantitatively into 5 mL vials and neutralized with 0.5 mL of 5 mM NaOH. The fatty acid methyl esters (FAME) were converted to the non-polar phase by the addition of 1 mL of n-hexane and vigorous shaking. The total lipids and FA profiles were determined by gas chromatography/flame ionization detection (GC/FID) analysis. GC analysis of FAME was carried out on a TRACETM 1300 Gas Chromatograph (Thermo Fischer Scientific, USA) equipped with a flame ionization detector, an Al 1310 autosampler and a Zebron ZB-FAME column (30 m, 0.25 mm, 0.20 μm, Phenomenex, USA). FAME were identified using commercial standard Supelco 37 Component FAME Mix (Sigma Aldrich). The internal standard method

was used for quantification via the addition of heptadecanoic acid (Sigma Aldrich) into the transesterification mixture in concentration 0.5 mg mL^{-1}. Chromatography data were evaluated using Chromeleon software 7.2.

2.5.3. Carbon biofixation

Carbon biofixation (g_{Carbon} L^{-1} d^{-1}) was quantified from biomass productivity (g_{DW} L^{-1} d^{-1}) and carbon content (g_{Carbon} g_{DW}^{-1}) in *P. engadinensis* KASU1 biomass, which was determined by FLASH 2000 CHNS/O Analyzer (Thermo Fisher Scientific, USA).

2.5.4. Statistical analysis

To evaluate the effect of individual cultivation conditions on the content and productivity of FA and carotenoids, one-way ANOVA was performed, if all necessary assumptions such as normal distribution and homogeneity of variance were fulfilled. Kruskal-Wallis test was used otherwise (Supplementary Table S2, S4). As far as multiple effect analysis was concerned, assumptions for factorial ANOVA were not fulfilled for any of the independent variable (Supplementary Table S3). To evaluate multiple effect analysis by nonparametric techniques, Aligned Rank Transform (ART) method [33] was adopted. The data transformation was done using ARTool software [34].

3. Results and discussion

3.1. Microalgae classification

P. engadinensis KASU1 exhibits a simple morphology. The round cells with diameter of approximately 5 μm contain multiple parietal chloroplasts (Supplementary Fig. S3). The cells have no apparent distinctive features and cannot be easily distinguished from other coccoid taxa like *Bracteococcus, Muriella, Chlorella* etc. For basic identification, sequencing of the widely sampled 18S rRNA gene marker was employed. The obtained partial 18S rRNA gene sequence was 99.9–100 % identical to the sequence of several strains of *Pseudomuriella engadinensis* recorded in GenBank (UTEX 57/SAG 221-3, UTEX 58/SAG 221-4). Subsequent phylogenetic analysis (Fig. 1) unambiguously confirmed its placement within the *Pseudomuriella* clade [35].

3.2. P. engadinensis KASU1 growth and biomass productivity

Growth of *P. engadinensis* KASU1 was characterized under a range of light intensities (100–1200 μE m^{-2} s^{-1}), CO_2 concentrations (450–10,000 ppm), temperatures (15–25 °C), and nitrate concentrations in the BG-11 cultivation medium (1.76–17.6 mM, see Supplementary Table S1 for details). The biomass productivity was ranging between 0.2 and 0.85 g_{DW} L^{-1} d^{-1}, and the specific growth rates in the exponential phase ranged between 0.24 and 1.57 d^{-1}, which corresponded with generation (doubling) time of *P. engadinensis* cells between 2.9 and 0.4 days.

The initial growth characterization under a range of CO_2 concentrations and light intensities revealed growth saturation [36,37] under 600 μE m^{-2} s^{-1} and 3000 ppm CO_2 in the bubbling gas (Fig. 2). The evaluation of FA and carotenoids production (as described in Section 3.3) was therefore performed under 3000 ppm CO_2.

Throughout all experiments, the maximal biomass content in *P. engadinensis* culture after 7 days of cultivation was 4.3 g_{DW} L^{-1}. The maximal biomass productivity was determined after 2 days of cultivation as 0.85 g_{DW} L^{-1} d^{-1} (Supplementary Table S1). The maximal specific growth rate and the maximal biomass productivity were identified under distinct light intensities (1200 and 600 μE m^{-2} s^{-1}, respectively). This is consistent with recent works showing that these parameters are

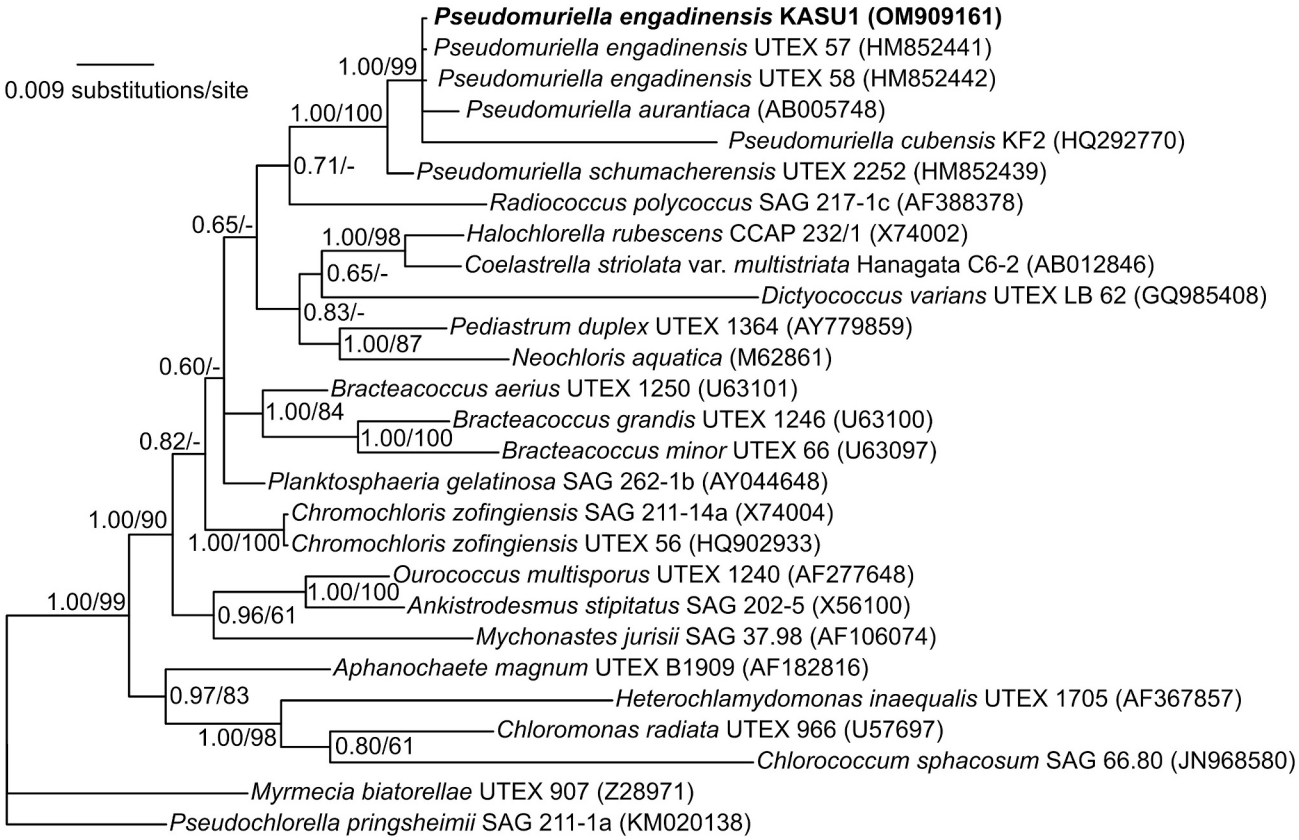

Fig. 1. Taxonomic placement of the strain KASU1. The strain clustered unambiguously within the genus *Pseudomuriella* with a high branch support (1.00/99), and its partial 18S rRNA gene sequence was identical to *P. engadinensis* SAG 221–3/UTEX 57. Label of the newly acquired sequence is printed in bold font. Nodes are decorated with branch supports in the following shape: Bayesian posterior probability/Maximum Likelihood bootstrap value.

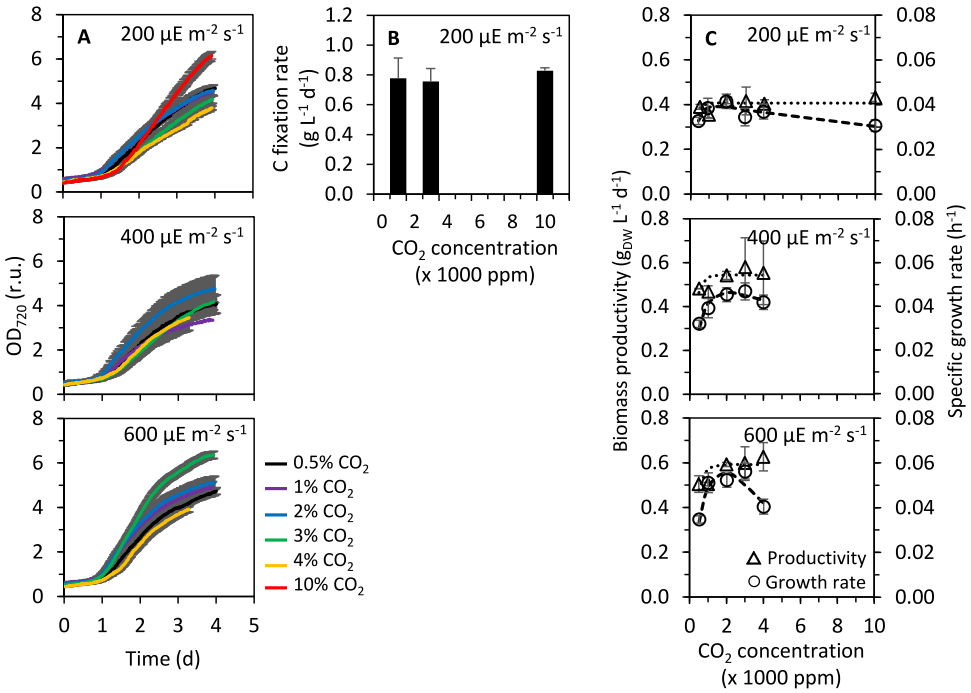

Fig. 2. Biomass growth, productivity (A, C) and CO_2 fixation rate (B) of *P. engadinensis* at 25 °C with 17.6 mM N in BG-11 cultivation medium and under 200–600 µE m^{-2} s^{-1} of white light and 450–10,000 ppm CO_2. Growth curves (A) were calculated from OD_{720} signal, as measured by MC 1000-OD and corrected according to Eq. (1). Carbon fixation rate was calculated only under 200 µE m^{-2} s^{-1}. Values represent averages (n = 2–15), error bars represent standard deviations (A) or standard errors (B, C). The dotted and dashed lines in panel C represent extrapolation of biomass productivity and specific growth rate by exponential regression model [64]. Further details on the cultivation conditions and culture productivities are provided in Supplementary Table S1.

not directly proportional [38].

P. engadinensis KASU1 is by far not the fastest growing alga; the fastest division time of 9.6 h observed in this study is still far from division time about 1.5 h in *Chlorella ohadii,* isolated from desert sand crust in Israel [9]. However, *P. engadinensis* KASU1 reached productivities comparable or even higher than other strains isolated from freshwater habitats [8,22]. However, the culture productivity strongly depends on the cultivation setup, and with the optimized setup that prevents nutrient and light limitation, even higher biomass productivity can be achieved (up to 10 g_{DW} L^{-1} d^{-1} [39]).

Optimal conditions for growth of *P. engadinensis* KASU1 were identified as 600 µE m^{-2} s^{-1}, 3000 ppm CO_2, 8.8–17.6 mM N and 25 °C (pH ranged between 6.85 and 7.55 under these conditions; see Supplementary Table S1 for details). Optimal light intensity higher than 400 µE m^{-2} s^{-1} and CO_2 concentration higher than 400 ppm are generally consistent with growth optima of other coccal green microalgae [40,41]. The specific value of 3000 ppm CO_2 has even been identified as optimal for growth of several microalgae strains [17,22,41]. The use of elevated CO_2 concentrations is attractive in terms of integration of waste gasses into microalgae production technology [17]. From this perspective, no significant decrease in carbon bio-fixation capability even under 10 % CO_2 (Fig. 2B) provides an important finding of suitability of *P. engadinensis* KASU1 strain for the combined applications of nutraceutical production and waste gases treatment.

3.3. Detected valuable compounds

An overview of all detected pigments and FA in *P. engadinensis* KASU1 biomass is summarized in Table 1. In total six carotenoids, seven FA and two chlorophylls were identified in the biomass extracts. Carotenoids with the highest biomass content were β-carotene and lutein (peak content 4.9 mg g_{DW}^{-1} and 3.8 mg g_{DW}^{-1}, respectively; Table 1). The most abundant FA were oleic acid (C18:1n-9) and γ-linolenic acid (C18:3n-6), with detected peak contents of 195.5 mg g_{DW}^{-1} and 81.9 mg g_{DW}^{-1}, respectively (Table 1).

β-carotene, lutein and astaxanthin are carotenoids beneficial for human health; they are important in preventing degenerative muscle disease and they reduce the risk of stroke and heart attack [17,42]. While β-carotene and astaxanthin are commercially produced by *Dunaliella salina* and *Haematococus pluviatis* [42], lutein is not yet commercially harvested from microalgae, despite its high concentrations compared to marigold petals that are traditionally used for its

Table 1
Overview of the detected compounds in *P. engadinensis* KASU1 biomass and respective peak concentrations throughout all experiments in this study. Together with contents, particular cultivation conditions are listed. MUFA: mono-unsaturated fatty acids, PUFA: poly-unsaturated fatty acids. Both MUFA and PUFA are expresses relative to total FA content.

Detected compound	Maximum content obtained in this work	Light intensity	N content	Temperature
	($mg\ g_{DW}^{-1}$; MUFA, PUFA: r.u.)	(µE m^{-2} s^{-1})	(mM)	(°C)
Pigments				
β-carotene	4.9	100	17.6	20
Lutein	3.8	100	17.6	25
Neoxanthin	1.3	100	17.6	25
Astaxanthin	1.3	600	8.8	20
Violaxanthin	0.4	100	8.8	20
Lycopene	1.3	100	17.6	25
Chlorophyll A	23.1	100	17.6	25
Chlorophyll B	13.7	100	17.6	20
Fatty acids				
MUFA	49.6	600	8.8	20
PUFA	52.8	100	17.6	20
Myristic acid (C14:0)	1.7	100	8.8	20
Palmitic acid (C16:0)	77.3	600	8.8	25
Palmitooleic acid (C16:1)	5.9	600	8.8	20
Stearic acid (C18:0)	42.9	600	8.8	25
cis-9-Oleic acid (C18:1n-9)	195.5	600	8.8	25
Linoleic acid (C18:2n-6)	35.8	600	8.8	25
γ-Linolenic acid (C18:3n-6)	81.9	600	8.8	25

production [17]. Lutein content in microalgal biomass typically ranges from 1.2 to 7.9 mg g_{DW}^{-1} [18]. The maximum lutein content in *P. engadinensis* KASU1 of 3.8 mg g_{DW}^{-1} as obtained in this study fits well to this range.

The most abundant FA determined in *P. engadinensis* KASU1 biomass, cis-9-Oleic acid (C18:1n-9), is common in many microalgae. Similarly, palmitic acid (C16:0) and stearic acid (C18:0) widely occur in Chlorophyta [43]. An important finding in *P. engadinensis* KASU1 is the detection of γ-Linolenic acid (C18:3n6) which is an essential PUFA, important for the prevention of cardiovascular diseases. γ-Linolenic acid was detected in several microalgae. However, its content was ranging only between 2.8 mg g_{DW}^{-1} in *Chlorella vulgaris* and19.2 mg g_{DW}^{-1} in the cyanobacterium *Arthrospira* [44]. In this study, an exceptionally high C18:3n6 content of 81.9 mg g_{DW}^{-1} was achieved, four times higher than the maximal content reported for microalgae so far.

3.3.1. *Effect of nitrogen concentration on fatty acids and carotenoids productivity*

After the initial growth characterization in full BG-11 medium (Fig. 2), the production of biomass, FA and carotenoids was tested under reduced N content (Fig. 3) under 600 μE m^{-2} s^{-1} 3000 ppm CO_2 and at 25 °C (Supplementary Table S1). N limitation led to an increase in FA content in *P. engadinensis* KASU1 biomass. The highest FA content was detected under 8.8 mM and 4.4 mM N (Fig. 3C). Since the biomass productivity under 4.4 mM N was reduced significantly (Fig. 3B), the optimal N concentration for FA production was identified as 8.8 mM. The FA with 18C (C18:0, C18:1n-9, C18:2n-6, C18:3n6) were identified as N-dependent (ANOVA / Kruskal-Wallis test: $p < 0.05$), whereas C14:0, C16:0 and C16:1 FA were N-independent ($p > 0.05$; Supplementary Table S2). Similarly, the PUFA content was increasing with N reduction (8.8 mM and 4.4 mM N), while the monounsaturated FA (MUFA) were nitrogen-independent (see Supplementary Table S2 for further details).

The FA content increase under N limitation is generally consistent with previous works [45]. Also, the increased accumulation of unsaturated FA such as C18:1 and C18:2n6 under N limitation was reported previously [46]. However, the FA profiling is species-specific [47], which underlines the importance of addressing the optimization of cultivation conditions for each strain individually.

Contrary to FA, the N limitation led to the reduction of carotenoid synthesis. The highest carotenoids content (1.36 mg g_{DW}^{-1}) was detected in full BG-11 medium (Fig. 3D). Four carotenoids were identified as N-dependent: lutein, β-carotene, neoxanthin, and violaxanthin (ANOVA / Kruskal-Wallis test: $p < 0.05$; Fig. 3D). The decrease of carotenoids under N limitation is consistent with other microalgae [22]. Nitrogen is considered as a key nutrient for cell growth. Under N limitation, microalgae prefer to accumulate high-energy compounds, and can even degrade carotenoids and reallocate the available carbon towards the synthesis of FA [22]. In addition, N limitation leads to the degradation of photosystems which is related to the degradation of structural carotenoids along with chlorophyll molecules [48]. Therefore, to optimize both FA and carotenoids productivity, the interplay between growth retardation, carotenoids degradation and FA synthesis needs to be considered. Since the productivity of carotenoids and biomass was the highest under 17.6 mM N and the productivity of FA was the highest under 8.8 mM N, further tests were performed under these two N concentrations.

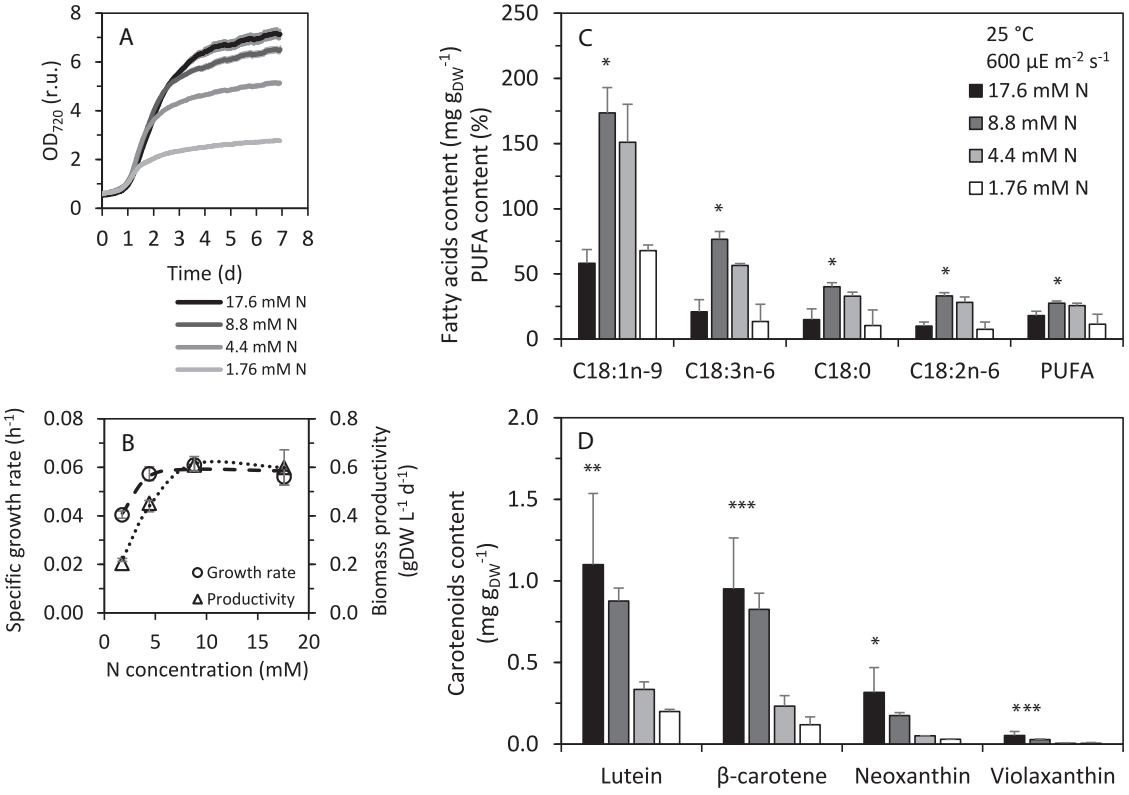

Fig. 3. Growth curves (A), the specific growth rates in the exponential phase, biomass productivities (B) and the content of fatty acids (C) and carotenoids (D) in the *P. engadinensis* KASU1 biomass under reduced nitrogen content in the BG-11 cultivation medium (1.76–17.6 mM). During the cultivations, the light intensity was set to 600 μE m^{-2} s^{-1}, temperature to 25 °C and CO_2 concentration to 3000 ppm. Data points represent averages (n = 9–28), error bars represent standard errors (A, B: growth rates) or standard deviations (B: productivity; C—D). The asterisks in panels C—D mark N-dependent FA and carotenoids with $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). Only N-dependent FA and carotenoids are shown, rest of the detected compounds (as summarized in Table 1) was identified as N-independent (see Supplementary Table S2 for details). The dashed and dotted lines in panel B represent data extrapolation by exponential regression model [64]. Further details on the cultivation conditions and culture productivities under reduced N content are provided in Supplementary Table S1.

3.3.2. Effect of light intensity on fatty acids and carotenoids productivity

The growth rates and composition of *P. engadinensis* KASU1 biomass were dependent on the intensity of cultivation light. Both biomass productivity and specific growth rates in the exponential phase were limited under 100 μE m^{-2} s^{-1} and saturated under 600 μE m^{-2} s^{-1}; further light intensity increase to 1200 μE m^{-2} s^{-1} did not lead to an increase in the growth rate, and it even reduced the biomass productivity (Fig. 4A-B). Therefore, evaluation of the biochemical composition of *P. engadinensis* KASU1 was performed under growth-limiting (100 μE m^{-2} s^{-1}) and growth-saturating light intensities (600 μE m^{-2} s^{-1}).

Light intensity affected the content of FA and carotenoids (Fig. 4C). The content of PUFA was enhanced under light limitation (up to 52.8 % of all FA; Table 1), in line with the reduction of MUFA (Fig. 4C). However, considering significantly higher biomass productivity under saturating light intensity (Fig. 4A-B), the highest production of light-dependent FA was detected under 600 μE m^{-2} s^{-1} (Fig. 5C). In line with our results, many strains increased PUFA content under low light [43], and total FA productivity under high light, even though specific differences were detected between individual FA and strains [19,43]. The optimization of FA and especially PUFA production by microalgae therefore needs to reflect the trade-off between high productivity of algae biomass under increased light intensity and high FA/PUFA content under low light [49].

The content of carotenoids (β-carotene, Lutein, Violaxanthin, Neoxanthin) together with Chlorophyll A and B was higher under 100 μE m^{-2} s^{-1} compared to 600 μE m^{-2} s^{-1} (Fig. 4D). The ratio of chlorophyll A:B varied between 0.55 and 2.05 (Supplementary Fig. S4), which is in agreement with previously reported values in algae [50]. Similar to the production of FA, the recommended optimal light intensities for carotenoids production vary from study to study. Lutein synthesis is typically enhanced under low light; as a part of the light harvesting antennas its content increases in order to improve light capture efficiency and to compensate for light limitation [51]. This applies also for *P. engadinensis* KASU1 (Fig. 4D). However, lutein accumulation also depends on the cultivation light wavelength, and is also predetermined by the composition of photosynthetic pigments that differ based on the phylogenetic position of particular algae [52]. It is therefore assumed that both the optimal light intensity and wavelength for lutein accumulation are strain-dependent, and detailed optimization towards lutein and other carotenoids production is necessary to perform for each studied strain [18].

3.3.3. Effect of temperature on fatty acids and carotenoids productivity

During the evaluation of biomass, FA and carotenoids production, *P. engadinensis* KASU1 was cultivated at 20 °C and 25 °C. The specific growth rates in the exponential phase were strongly affected by temperature (Fig. 4B, Fig. 5B). However, after 7 days of cultivation during linear and stationary growth phases, cultures at both 20 °C and 25 °C reached similar densities (however, still significantly different: $p < 0.05$; Fig. 4A, Fig. 5A), which mitigated the effect of temperature on the final

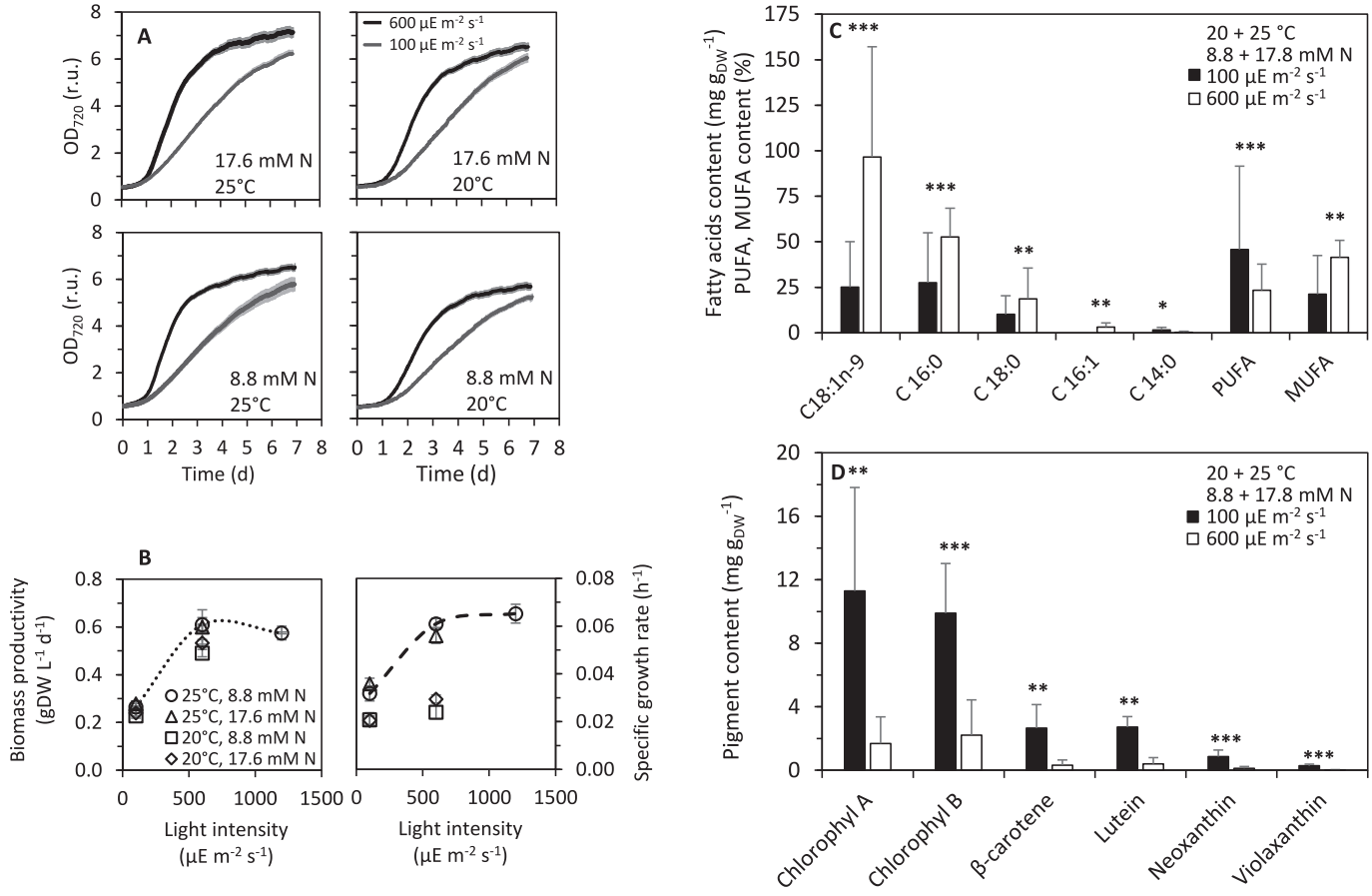

Fig. 4. Growth curves (A), biomass productivity, specific growth rate in the exponential phase (B) and the content of fatty acids (C) and carotenoids (D) in the *P. engadinensis* KASU1 biomass under growth-limiting and saturating light intensities (100 and 600 μE m^{-2} s^{-1}, respectively). Data points represent averages (n = 8–28), error bars represent standard errors (A, B: growth rates) or standard deviations (B: productivity, C—D). The asterisks in panels C—D mark light-dependent FA and carotenoids with $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). Only light-dependent FA and carotenoids are shown, rest of the molecules (as summarized in Table 1) was identified as light-independent (see Supplementary Table S3 for details). The dotted lines in panel B represent data extrapolation by exponential regression model [64]. Further details on the cultivation conditions and culture productivities under reduced N content are provided in Supplementary Table S1.

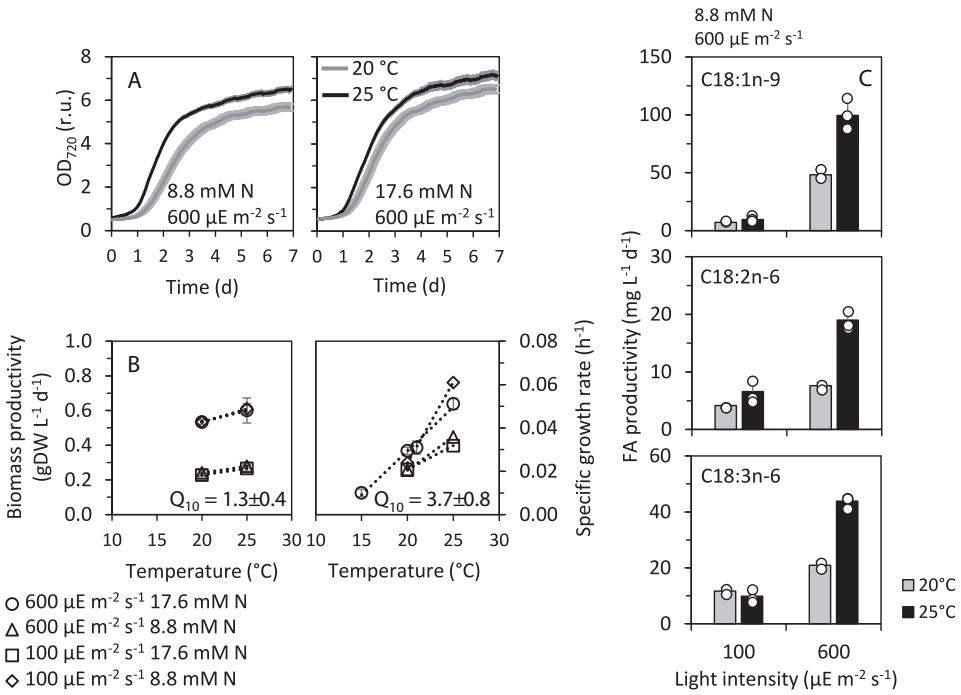

Fig. 5. Growth curves (A, D), biomass productivity, specific growth rates in the exponential phase (B) and productivity of fatty acids (C, E) by *P. engadinensis* KASU1 under constant temperature of 20 °C and 25 °C. Data points represent averages (n = 2–28), error bars represent standard errors (A, B: growth rates) or standard deviations (B: productivity). Statistical significance of carotenoids and fatty acids productions under 8 mM N and constant 20 / 25 °C was not tested due to limited amount of data points under some conditions (C; n = 2–3). Instead, measured data points (white dots) and averages (bars) are shown. The dashed lines in panel B represent data extrapolation by linear regression model.

biomass productivity (Fig. 5B).

At 20 °C, PUFA increased significantly at the expense of saturated FA, mainly due to C18:3n-6 increase, which alone, however, was not statistically significant ($p > 0.05$; Supplementary Table S3). Even though the PUFA concentration in the *P. engadinensis* KASU1 biomass increased at 20 °C (Supplementary Table S3), the productivity of C18:1n-9, C18:2n-6 and C18:3n-6 was higher at 25 °C at growth-saturating light intensity (Fig. 5C), due to the increased productivity of biomass (Fig. 5A-B). From carotenoids, only neoxanthin was affected by temperature (see Supplementary Table S3 for further details). Under light limitation the temperature increase did not lead to the increase in productivity of FA or carotenoids (Fig. 5C).

In addition to addressing the effect of constant temperature, the effect of temperature shifts towards the FA and carotenoids productivity was tested (Supplementary Fig. S4, Supplementary Table S4). To test the production of FA and carotenoids in conditions close to the natural occurrence of the strain, the temperature range (15–25 °C) was based on the conditions measured on the sampling site during summer 2019 (Supplementary Fig. S1). During the shift tests, the *P. engadinensis* KASU1 cultures were let to acclimate to constant temperature during the batch growth for two days, after which the temperature was shifted by 5 °C (both descending and ascending; Supplementary Fig. S4). The ascending temperature led to slight (but still significant) increase in the content of two carotenoids (lycopene, neoxanthin), and vice versa, the descending temperature led to slight but significant increase in the production of FA (C18:1n-9, C18:2n-6, MUFA; Supplementary Fig. S4). Other carotenoids ad FA were not affected by the temperature shift. We note that the highest carotenoids and FA productivities were always achieved under constant temperatures (Fig. 5C).

Temperature drives reaction speed of the enzymatic reactions and therefore represents a crucial factor for growth. Temperature also causes shifts in FA profile [53]. The strategy of temperature shifts appears advantageous: in the initial phase, the biomass is allowed to accumulate under optimal growth temperature, and in the second phase, synthesis of the product of interest (such as PUFA) is enhanced under reduced temperature. Similar bi-phasic cultivation approach has been successfully tested for the production of secondary metabolites [54].

The results of temperature characterization are consistent with the previous works; under N limitation an increase of C18:2n-6 and C18:3n-6 has been reported at 25 °C [55]. N limitation leads to a drop in the photosynthetic activity, due to an adverse effect on PSII [56]. This, together with temperature sensitivity of the nitrate uptake [57] suggests that for FA production optimization, either biphasic cultivation, or cultivation under optimal temperature is required. This is consistent with previous works [55]. Identical to other factors tested in this study, the simultaneous effect of temperature shift and N-limitation on the FA accumulation is still an understudied phenomenon, which remains to be addressed for many algal strains [56].

3.4. Potential for applications

Microalgae are considered as a promising source of valuable food and feed ingredients. The microalgae are a highly diverse group of microorganisms. Therefore, the biochemical composition of microalgal biomass varies significantly between taxa, and even between similar strains of closely related species such as *Nannochloropsis* sp. [44]. In the biomass of *P. engadinensis* KASU1, lutein and β-carotene were the most abundant carotenoids (Table 1). The highest production of β-carotene was 0.8 ± 0.1 mg L^{-1} d^{-1}, about three to four times lower compared to *Tetradesmus acuminatus* [5] and *Dunaliella salina* [58]. Similarly, maximal lutein production of 0.9 ± 0.1 mg L^{-1} d^{-1} was about eight times lower than in *Chlorella minutissima* [18]. From this perspective, *P. engadinensis* KASU1 does not appear as exclusive strain for carotenoids production. However, carotenoids can represent an interesting by-product during production of more expressed metabolites such as FA.

Productivities of FA were as high as 100 ± 13 mg L^{-1} d^{-1} (C18:1n-9). Reports of individual FA productions by microalgae are scarce. Del Río and colleagues reported a C18:1n-9 productivity of 88 mg L^{-1} d^{-1} for a microalga *Pseudokirchneriella subcapitata* and 47 mg L^{-1} d^{-1} for *Chlorococcum oleofaciens* [45], which is 88 % and 53 % of the productivities obtained here, respectively. The productivity of C18:2n-6 (19 ± 2 mg L^{-1} d^{-1}), another FA important for human diet, was matching the previous reports of *Chlorococcum oleofaciens* and even overcomes production in *Pseudokirchneriella subcapitata* [45].

However, the most important finding was the detection of γ-linolenic acid (C18:3n-6) – essential health benefiting PUFA [59]. Many studies have shown the anti-inflammatory effects of C18:3n-6 and its important role in the prevention of atherosclerosis [59,60]. The obtained C18:3n-6

productivity of 44 ± 2 mg L^{-1} d^{-1} is the highest productivity reported for microalgae up to date; previous works reported productivities between 5 and 37 mg L^{-1} d^{-1} by a variety of strains [45,61]. C18:3n-6 is present in the seed oils of wild plants such as *Primula* spp., *Echium* spp., *Cannabis sativa*, and *Borago officinalis*. However, only low amounts of C18:3n-6 are produced from these plants, and many studies therefore aim to enhance PUFA accumulation by using transgenic plants. In contrast to the production in plants, the production of C18:3n-6 by microalgae such as *P. engadinensis* KASU1 has a potential to provide a stable yield throughout the whole year under well-defined cultivation conditions. Furthermore, the microalgae production can be operated on a non-arable land that does not compete with food production [62].

Additional value of *P. engadinensis* KASU1 is the ability to grow under high CO_2 concentrations (Fig. 1); an attribute important for CO_2 sequestration. Air enriched with CO_2 is often used for microalgae cultivation [22]. Recent reports have proven the concept of utilization of industrial waste gases, rich of CO_2, as an inexpensive carbon source [63]. Treatment of the waste gasses, produced during incineration processes as a part of industrial production, then provides additional advantage and another added value of the industrial microalgae production.

4. Conclusions

In this work, the underexplored potential of the intermittent streams for the isolation of microalgae with biotechnological potential is demonstrated. *Pseudomuriella engadinensis* KASU1 was identified as the fastest-growing microalgae strain present in the dry riverbed sample. The intermittent streams provide a strong pressure for the synthesis of anti-oxidative compounds such as carotenoids as well as for shifting the FA profiles towards the synthesis of PUFA. Considering generally high productivities of all bioactive compounds, and even the highest reported productivity of γ-Linolenic acid, the strain *P. engadinensis* KASU1 is a perfect candidate for the production of bioactive substances, combined with the simultaneous industrial flue gases treatment.

CRediT authorship contribution statement

Kateřina Sukačová: Conceptualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Visualization, Writing – original draft. **Martin Szotkowski:** Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing. **Petr Pařil:** Investigation, Writing – review & editing. **Jan Mareš:** Investigation, Formal analysis, Data curation, Visualization, Writing – review & editing. **Michal Touš:** Formal analysis, Data curation, Writing – review & editing. **Daniel Vícha:** Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing. **Marek Polášek:** Writing – review & editing. **Ivana Márová:** Writing – review & editing. **Tomáš Zavřel:** Conceptualization, Formal analysis, Data curation, Visualization, Writing – original draft.

Declaration of competing interest

The authors have no conflicts of interest to disclose and have all approved this submission.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103055.

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