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Research Article

LIGHT INTENSITY AND NITROGEN EFFECTIVELY CONTROL EXOPOLYSACCHARIDE PRODUCTION BY THE GREEN MICROALGA *BOTRYOCOCCUS BRAUNII* (TREBOUXIOPHYCEAE)

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Summary: The production of exopolysaccharides (EPS) in the green microalga *Botryococcus braunii* strain CCALA 220 (race A), at different nitrate concentrations and light intensities, was investigated. EPS content was strongly dependent not only on the nitrogen concentration in the cultivation medium, but also on the intensity of light. EPS accumulation in the medium was negligible below light intensities of 450 μmol m^{-2} s^{-1}, regardless of the nitrogen concentration. At higher light intensities (650-950 μmol m^{-2} s^{-1}), EPS production was dependent on the nitrogen concentration in the cultivation medium. The optimal concentration of nitrogen, supplied as potassium nitrate, was 6 mM for EPS production, whereas it was 24 mM for biomass growth or chlorophyll concentration. Higher light intensities suppressed growth as well as EPS production, probably due to photoinhibition. In addition, the ultrastructure of cells grown under various conditions dramatically changed. Hydrocarbon-producing cells had a well-developed chloroplast, which was in contrast to EPS producing cells that were without a prominent chloroplast but had a large structured vacuole. In *B. braunii* CCALA 220, the optimal conditions of light and nitrogen for EPS production were 950 μmol m^{-2} s^{-1} and 6 mM nitrate, respectively, under which EPS approached 2 mg mL^{-1}. These conditions were accompanied by significant ultrastructural changes.

Keywords: *Botryococcus braunii*; EPS; biomass; light intensity; nitrate concentration; ultrastructure.

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INTRODUCTION

Polysaccharides isolated from algae and cyanobacteria are listed as substances that can be produced in the biotechnology industry, mostly for utilization in food processing such as for viscosifiers, flocculating agents or lubricants (Borowitzka, 1988). High-molecular-weight water-soluble polymers may be desirable as industrial emulsifiers and thickeners (Atobe et al., 2015). Polysaccharides may also have medicinal applications, *e.g.* as

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anti-HIV agents (Schaeffer & Krylov, 2000) or as antiadhesive therapies against *Helicobacter pylori* infections, as tested *in vitro* (Guzman-Murillo & Ascencio, 2000). Antiviral substances of polysaccharide nature have been isolated from brown (Zhu et al., 2003), green (Lee et al., 2004), red algae (Haslin et al., 2001), dinoflagellate microalgae (Yim et al., 2004) and from cyanobacteria (Kanekiyo et al., 2005). Algal polysaccharides also have antibacterial (Reichelt & Borowitzka, 1984), antitrombin (Trento et al., 2001), anti-inflammatory (Matsui et al., 2001), anti-cough (Nosáľová et al., 2011), anti-ulcer (Nagaoka et al., 2000), anti-tumor (Itoh et al., 1993; Kamble et al., 2018) and antioxidant (Hu et al., 2001; Fimbres-Olivarria et al., 2018; Kamble et al., 2018) properties. Most polysaccharides have to be extracted from algal cells or thalli, so exopolysaccharides (EPS) released by some living algae into their environment are of great interest due to their ease of production and harvesting. Various cyanobacteria and microalgae are able to produce EPS, but few microalgal polysaccharides are used commercially. EPS synthesized by *Porphyridium cruentum* Nägeli is considered to be commercially important (Anderson & Eakin, 1985); more recently bioactive effects of EPS from the green microalga *Dictyosphaerium chlorelloides* (Nauman) Komárek & Perman (Halaj et al., 2018) ant its prospective biotechnological production were investigated.

The green colonial microalga, *Botryococcus braunii* Kützing has been recognized as a prospective photoautotrophic microorganism for biotechnological applications, mostly due to the production of large amounts of high-energy hydrocarbons excreted from the cell to the environment (Largeau et al., 1980; Casadevall et al., 1985; Griel et al., 2015; Jackson et al., 2017). There are many *B. braunii* isolates being dividing into three main races (A, B, and L) according to the prevalent hydrocarbon species, i.e. n-alkadiens, botryococcenes, and lycopene, respectively (for review see Banerjee et al., 2002). *B. braunii* races are correlated with their 18S rRNA molecular phylogenies, so the phylogenetic relationship of *Botryococcus* strains appeared to be consistent with unique hydrocarbon biosynthetic pathways (Weiss et al., 2011; Kawachi et al., 2012). In addition to extracellular hydrocarbons, some strains (mostly races A and B) also produced considerable amounts of intracellular triacyglycerols (Moutel et al., 2016). These features of *B. braunii* have attracted attention as microalga that could satisfy demands for an alternative source of biofuels (Ashokkumar & Rengasamy, 2012; Ranga Rao et al., 2012).

However, *B. braunii* is also capable of synthesizing substantial amounts of exopolysaccharides (EPS), as was first reported for race A (Casadevall et al., 1985). Compared to hydrocarbons and TAG however, the potential of *B. braunii* EPS has been largely neglected, although some information exists. The complex composition of its EPS depends on strain or race, although galactose is always the major component, followed by arabinose, uronic acids and also rhamnose and fucose (Allard

& Casadevall, 1990; Metzger et al., 1990; Atobe et al., 2015). In contrast to races A and B, glucose was detected in significant amounts in EPS from race L (Allard & Casadevall, 1990; Metzger et al., 1990). Polysaccharide production occurred over all growth phases, but higher rates of production were seen as the growth rate declined. This enhanced production is associated with the onset of nitrogen limitation in the culture medium (Banerjee et al., 2002). An interesting, unwanted, consequence of *B. braunii* EPS was reported recently. Water-soluble polysaccharides released from *B. braunii* race B reduced the recovery of hydrocarbons by thermal pretreatment of an algal slurry, and thus should be removed prior to hydrocarbon extraction (Atobe et al., 2015).

Some attention has been paid to determining culture conditions that enable maximum EPS production. The most important factors that have been documented are: i) culture strain (Fernandes et al., 1989); ii) temperature (Lupi et al., 1991); iii) nitrogen source (Lupi et al., 1994; Qin & Li, 2006); and iv) salinity (Bayona & Garcés, 2014). In addition, photoperiod (Lupi et al., 1994) and light intensity (Cepák & Lukavský, 1994) strongly influence EPS production.

However, littleis knownabout abiotic factors that affect growth and production of EPS in *B. braunii* and available data are rather heterogeneous and sometimes even controversial. The aim of the present investigation was, therefore, to combine a wide range of nitrate concentrations with varying light intensities to determine optimum conditions for maximum production of EPS in laboratory batch cultures.

MATERIAL AND METHODS

Organism and growth experiments

The microalga, *Botryococcus braunii* strain CCALA 220, race A, identical to SAG 807-2 (http://sagdb.uni-goettingen.de/detailedList.php?str_number=807-1) and CCAP 807/2 (https://www.ccap.ac.uk/strain_info.php?Strain_No=807/1), was obtained from the Culture Collection of Autotrophic Organisms (CCALA, http://ccala.butbn.cas.cz/en/botryococcus-braunii-kuetzing) in Třeboň, Czech Republic. The strain has been maintained on agar slants (2% in Zehnder medium, Staub 1961) at a light intensity of 23 μmol m^{-2} s^{-1} PAR, photoperiod 12:12 h (L:D), and at a temperature of 12°C. Light intensity was measured using a PU 550 digital lux-meter (Metra, Blansko, Czech Republic) equipped with a PAR sensor calibrated according to the LI-185 quantum sensor (LI-COR, Lincoln, USA). Batch cultures were grown under continuous light of various intensities (100, 230, 450, 950 and 1400 μmol m^{-2} s^{-1}) and at a temperature of 28°C in Tamyia flasks (500 mL). Fluorescent cool white lamps were used as a light source. Cell suspensions were bubbled with air supplemented with 2% carbon dioxide (v/v), simultaneously providing mixing for the cultures. The cultures were gradually adapted to the higher light as follows. To prepare inocula for experiments culture of *B. braunii* was transferred from agar slant into liquid Zehnder medium and grown under conditions described above. Light intensity was set-up at 100 μmol m^{-2} s^{-1} and gradually increased by 100 μmol m^{-2} s^{-1} each 2-3 days until

the desired light intensity for batch cultivation experiments was reached. For the nutrient experiments, an inorganic nutrient solution after Zehnder was fortified with various concentrations of nitrogen as potassium nitrate (0.1, 0.9, 6.0, and 24 mM).

Analyses

EPS were determined gravimetrically as follows. Ten mL samples were diluted with 50 mL of distilled water and, after centrifugation, the supernatant was collected. The pellet was resuspended in 10 mL of distilled water, recentrifuged and the supernatant was combined with the previous one. Total supernatants were mixed with double the volume of 2-propanol and the precipitated EPS were passed through a pre-weighted Whatman GF/C filter, dried at 80°C for 24 hours, and weighed on an analytical Mettler Toledo XSE balance (Columbus, OH, USA). The cells (pellet) were collected on a pre-weighted Whatman GF/C filter, dried to constant weight at 105°C, and weighed on an analytical balance as above. The DW of biomass was then calculated by adding the pellet and EPS dry weights. Total chlorophyll content was determined spectrophotometrically as follows. Ten ml of cell suspension was mixed with 10-50 mL of phosphate buffer (pH 7.4), centrifuged and chlorophyll was extracted from the pellet using 80% acetone as described previously (Lukavský et al., 1979). Absorbances at wavelengths 646.6 and 663.6 nm were measured using a UV-1650PC spectrophotometer (Shimadzu, Japan). Total chlorophyll concentration was calculated according to Porra's equations (Porra, 2002).

Cultivation experiments and all growth and biochemical analyses were performed in triplicate. Statistically significant differences between treatments were computed by t-test using Statistica 12 (StatSoft, USA) and all graphs were created using Sigma Plot 11.0 (Systat Software, USA).

Transmission electron microscopy

Microalgal samples were preserved with 2% formaldehyde and washed in 0.2 M cacodylate buffer, pH 7.4. Afterwards, they were post fixed with 2% w/v OsO_4 and embedded in 2% w/v agar. Samples were dehydrated in an acetone series, transferred into Spur's resin (Fluka, Germany) and stained in two steps with uranyl acetate and lead citrate in ethyl alcohol. For observation, a PHILIPS SM 300 electron microscope (Amsterdam, Netherlands) was used and images were collected using a JSM-7401F camera (Jeol, Japan).

RESULTS AND DISCUSSION

Following 15-days of batch cultivation of *Botryococcus braunii* in Zehnder medium (6 mM nitrogen) at different light intensities, culture aliquots were harvested and cells were allowed to settle for an hour. Fig. 1a indicates distinct differences in cell substances resulting in different features of suspensions at various light intensities. While at the lowest light intensity (100 μmol m^{-2} s^{-1}) the cells settled down to the bottom of the test tubes, indicating a high specific density due to the starch accumulation, at medium light intensities (230 and 450 μmol m^{-2} s^{-1}) cells floated on the

surface of the medium, presumably due to their production of hydrocarbons. At the highest irradiance (950 μmol m^{-2} s^{-1}) the suspension viscosity was high and prevented cells from floating or settling, and cultures released a significant amount of polysaccharide into the medium (Fig. 1a). Similar changes in culture suspension in response to light intensity were previously described in *B. braunii* DROOP 1950/807-1 (Cepák & Lukavský, 1994), strain identical to CCALA 220. This ability of *B. braunii* to adjust to different growth conditions and produce various metabolites is also in agreement with results obtained by Dayananda et al. (2007) with the same *B. braunii* strain (UTEX LB 572, identical to CCALA 220). Although they applied a different experimental design (lower light intensity, longer treatment time, no carbon dioxide supplementation), the importance of light for EPS production was apparent, as continual light favored EPS over hydrocarbons compared to the 16:8 light:dark photoperiod (Dayananda et al., 2007). Similarly, higher volumetric and specific productivity of EPS was found in *B. braunii* cells grown under continuous illumination than those grown under cyclic illumination (Lupi et al., 1994).

Based on these trial results, we conducted further experiments to optimize the nitrogen concentration for EPS production. *B. braunii* was grown

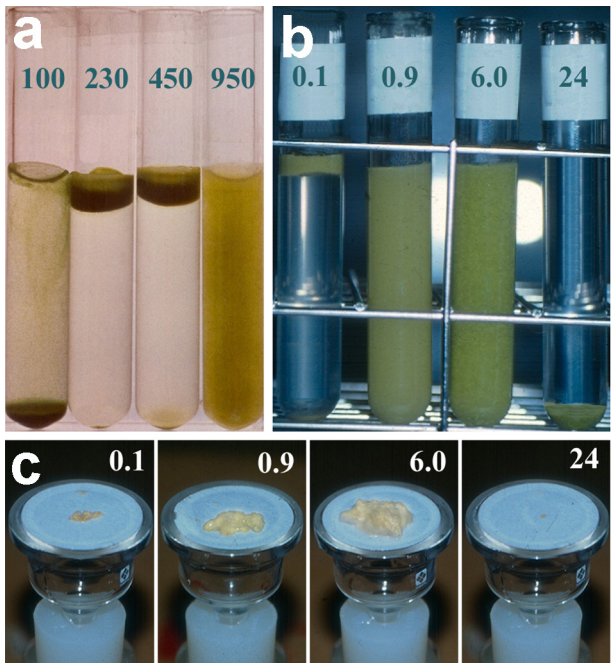

Figure 1. a. Cell suspensions of *B. braunii* grown at various light intensities (100-950 μmol m^{-2} s^{-1}) in Z medium (6 mM nitrogen) for 15 days. At low light intensity, cells of a high specific density settled on the bottom of the cultivation vessel, indicating a high starch content, whereas at medium light intensities, cells were lighter in weight due to hydrocarbons and floated on the medium surface. Cells grown at highest light intensity were immobilized in EPS. **b, c.** Cells grown at 950 μmol m^{-2} s^{-1} at various nitrate concentrations (0.1-24 mM). **b.** Medium nitrate concentrations favored EPS production – cells immobilized in EPS. **c.** EPS precipitated and filtered from equal volumes of suspensions.

at a light intensity (950 μmol m^{-2} s^{-1}) that was optimal for EPS production, but at various nitrate concentrations for 21 days. The suspension aliquots were allowed to settle and were evaluated as previously. The lowest nitrate concentration used (0.1 mM) favored hydrocarbon production, medium nitrate concentrations (0.9 and 6 mM) resulted in EPS production, while cells accumulated mostly starch at the highest nitrate concentration (24 mM) (Fig. 1b). When EPS were precipitated from aliquots of supernatants, and filtered (see Material and methods), 6 mM nitrate was shown to be the optimal concentration for EPS production under the above conditions (Fig. 1c).

Quantification of EPS showed that at the optimal nitrogen concentration (6 mM), up to 2 mg mL^{-1} EPS were produced, which represented approximately 40 % of the total biomass based on dry weight. Maximal values of 1.6-1.8 mg mL^{-1} EPS (Dayananda et al., 2007) and significantly lower values 0.25 and 0.55 mg mL^{-1} EPS (Metzger et al., 1990; Bayona & Garcés, 2014, respectively) have been previously reported from this strain. An extraordinarily high accumulation of EPS (4-4.5 mg mL^{-1}) was described in a *B. braunii* strain UC 58 (=ACOI 58), although a very long period of cultivation (7 weeks) at a light intensity of 250 μmol m^{-2} s^{-1} was used (Fernandes et al., 1991). Such different results indicate the importance of cultivation conditions over strain used for EPS production. About 0.7 mg mL^{-1} EPS were produced at a nitrate concentration of 0.9 mM, whereas negligible amounts of EPS were produced at limiting nitrate concentrations (0.1 and 24 mM) (Fig. 2). On the contrary, the optimal nitrogen

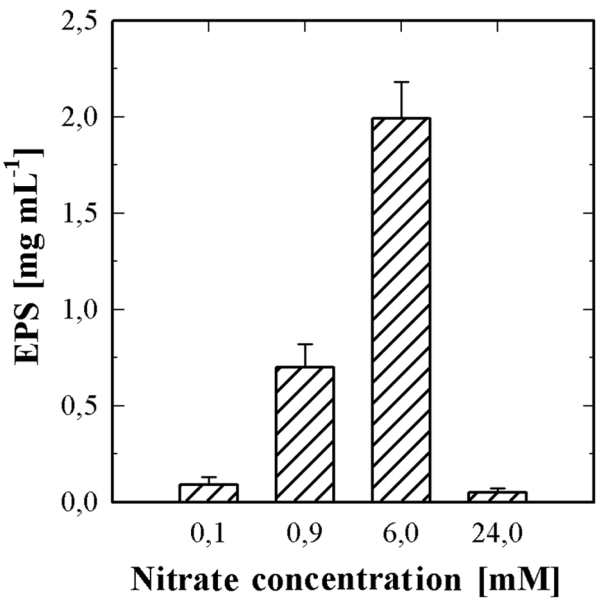

Figure 2. Effect of nitrogen on EPS accumulation in *B. braunii* batch cultivated at a light intensity of 950 μmol m^{-2} s^{-1} for 21 days. The cells preferred to produce EPS at medium nitrate concentrations. Data are means (±SD) of three replicates, asterisks indicate significantly similar values ($p>0.01$).

concentration for culture growth at 950 μmol m^{-2} s^{-1} was 24 mM, as shown from dry matter accumulation and the concentration of chlorophyll. The lower the nitrogen concentration, the lower was the dry weight or chlorophyll concentration (Fig. 3). At a nitrogen concentration of less than 0.9 mM, conditions became growth limiting to the culture. The ratio between dry matter and chlorophyll content decreased with increasing amount of nitrogen in the cultivation medium, so nitrogen clearly favored chlorophyll biosynthesis. Overall, the requirements for growth and EPS production were mutually exclusive, higher and medium nitrate concentrations favored biomass and EPS, respectively (compare Figs. 2 and 3). This finding partly corresponds to the result published by Lupi et al. (1991), who studied the effect of temperature on EPS production in *Botryococcus* strain ACOI 58. Contrary to our results, they concluded that the highest accumulation of EPS coincided with the optimum for growth, however they grew *Botryococcus* at lower light intensity (250 μmol m^{-2} s^{-1}) and at a single nitrogen concentration (2 mM, Lupi *et al*. 1991). The differences in results may be due to the fact that growth conditions for *Botryococcus* ACOI 58 were not adequately determined. Strain ACOI 58 accumulated high levels of EPS (4.5-5 mg mL^{-1}) at 250 μmol m^{-2} s^{-1} (Lupi et al., 1991; 1994), whereas our strain excreted negligible amount of EPS at similar light intensities (Fig. 4).

At the optimal conditions for growth (light intensity 950 μmol. m^{-2}. s^{-1}, 24 mM nitrate) we achieved a biomass of about 5.5 g L^{-1} DW (Fig. 3). This value is comparable with growth-optimized

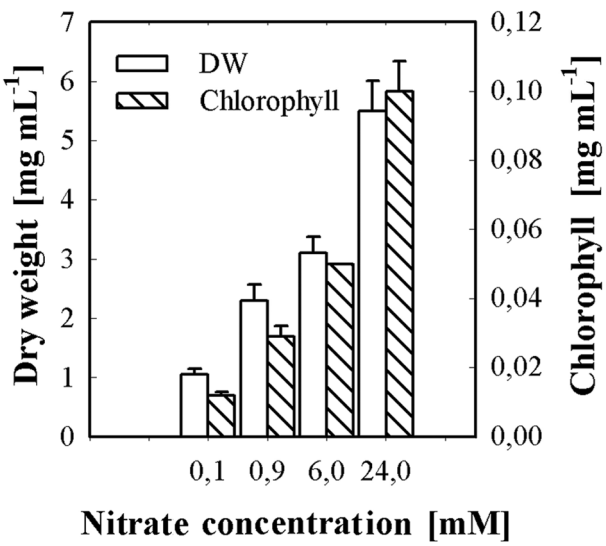

Figure 3. Effects of nitrogen on biomass and chlorophyll in *B. braunii* grown at a light intensity of 950 μmol m^{-2} s^{-1} for 21 days. Optimal nitrate concentration for growth was higher (24 mM) than for EPS accumulation (0.9 – 6 mM). Data are means (±SD) of three replicates, asterisks indicate significantly similar values ($p>0.01$).

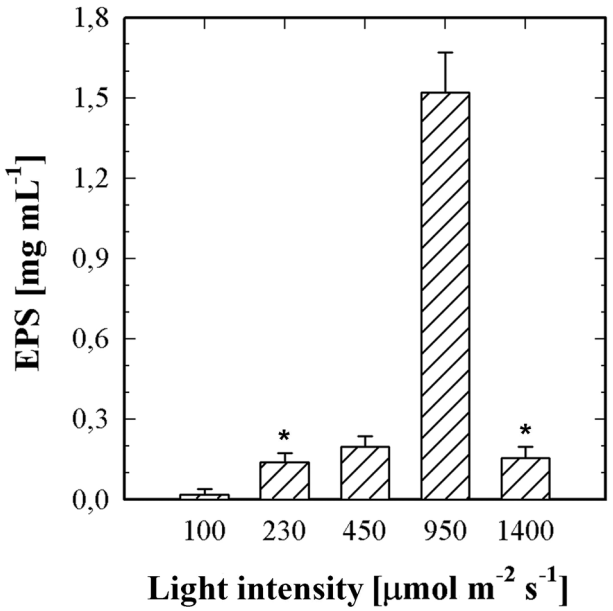

Figure 4. Effect of light intensity on EPS accumulation in *B. braunii* batch cultivated in medium containing 6 mM of nitrate for 15 days. The cells produced EPS at a higher light intensity (950 μmol m^{-2} s^{-1}), but too high light intensity inhibited their accumulation. Data are means (±SD) of three replicates, asterisks indicate significantly not different values ($p>0.01$).

cultures of B-race *B. braunii* cultured in a bubble column photobioreactor, where over 7 mg mL^{-1} DW was achieved after 25 days (Kojima & Zhang, 1999). Other authors have reported substantially lower biomass concentrations in this species; 1.0-3.7 g L^{-1} DW (Wolf et al., 1985; Metzger et al., 1990; Fernandes et al., 1991; Qin & Li, 2006; Dayananda et al., 2007; Bayona & Garcés, 2014). These differences can be explained by both the strain used and growth conditions. Usually the light intensity has not been high enough to achieve high biomass *e.g.* 60 μmol. m^{-2}. s^{-1}, 1 g L^{-1} DW (Bayona & Garcés, 2014), 250 μmol. m^{-2}. s^{-1}, 2 g L^{-1} DW (Fernandes et al., 1991), 470 μmol. m^{-2}. s^{-1}, 3.6 g L^{-1} DW (Metzger et al., 1990). Together with higher light intensities, supplementation with carbon dioxide has also been used to enhance growth of *B. braunii* (Metzger et al., 1990; Cepák & Lukavský, 1994; Kojima & Zhang, 1999). Previously it was shown that *B. braunii* CCALA 220 grew best in culture when aerated with a mixture of air and carbon dioxide (2% v/v), whereas air-lifted cultures (or air-lift with addition of bicarbonate) grew much slower (Cepák & Lukavský, 1994). We may conclude that carbon nutrition plays a significant role in algal growth and accumulation of various metabolites. To assess an importance of light for EPS accumulation, we applied a wide range of light intensities (100-1400 μmol m^{-2} s^{-1}) on cultures growing at the nitrate concentration optimal for EPS production (6 mM) for 15 days. The amount of EPS peaked at a light intensity of 950 μmol m^{-2} s^{-1} (1.6 mg mL^{-1}), whereas it decreased proportionally

to a reduction of light intensity. At the highest light intensity used (1400 μmol m^{-2} s^{-1}) however, EPS accumulation was also relatively low, probably due to the photoinhibition (Fig. 4). Previously *B. braunii* strain CHN 357 was studied from the aspect of lipid production. When this strain was cultivated at a light intensity of 920 μmol m^{-2} s^{-1} (optimal for our strain for EPS accumulation), no production of EPS was recorded (Qin & Li, 2006). There can be several reasons for this discrepancy. The metabolic pathway for EPS biosynthesis may be strain specific (and the optimal temperature is lower for strain CHN 357). The suspension was not supplemented with carbon dioxide, which is essential for higher growth rates. *B. braunii* CHN 357 was cultivated more than 4 weeks and the maximal biomass achieved was only 1 mg mL^{-1}. There is no information about the exact composition of the culture medium (especially nitrogen content). An SE (soil extract) cultivation medium of not defined composition was employed, which is normally used only for enrichment of an appropriate inorganic medium (Nichols, 1973). Hence some growth conditions (temperature, nutrients) were probably not adequate for EPS accumulation.

Varying nitrate concentrations in the medium had no effect on cell ultrastructure at the end of the experiment; only light intensity caused substantial ultrastructural changes. Cells growing at lower light intensities (up to 450 μmol m^{-2} s^{-1}) had the usual intracellular structures with a well-developed parietal chloroplast and were surrounded by hydrocarbon droplets of various sizes (Fig. 5a); their appearance resembled micrographs published in other ultrastructural studies (Largeau et al., 1980; Bailliez et al.,1988; Cepák & Lukavský, 1994). High light conditions were accompanied by profound changes of cell ultrastructure as a result of altered metabolism of storage compounds. After three weeks of cultivation at a light intensity of 950 μmol m^{-2} s^{-1}, the EPS producing cells showed ultrastructural reorganization of the protoplast (Fig. 5b) in comparison with cells grown under lower light intensities, regardless of nitrogen concentration (Fig. 5a). However, this state was reversible and when cells were grown further under lower light intensity, the cells reconstructed their chloroplasts and regenerated their "normal" cellular appearance (not shown). Within cells cultivated under high light conditions, a strongly reduced chloroplast was observed; the most conspicuous organelle was a large vacuole containing a system of membranes and lagoons. Cells were embedded in the polysaccharide, appearing as a compact less-electron dense matrix (Fig. 5b). Detailed analyses of *B. braunii* race B using quick freeze deep-etch electron microscopy revealed a fibrillar polysaccharide sheath covering the whole surface of cell colonies. Polysaccharide fibrils extended from the retaining cell walls (Weiss et al., 2012) and they appeared to be a structural component as they remained on the empty cell walls ("shells") after extraction (Weiss et al., 2012). The relationship between polysaccharide fibrils and potentially soluble EPS was, however, not studied.

Our results demonstrate that *B. braunii* CCALA 220, recognized as low EPS-yielding strain can, under

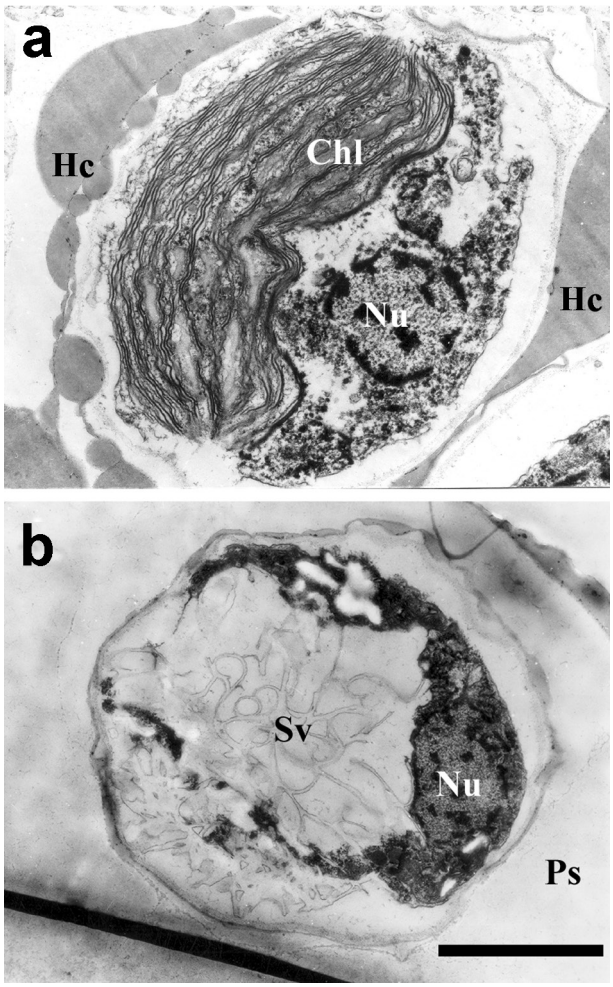

Figure 5. TEM images of *B. braunii* cells grown under various conditions for 21 days. **a.** "Normal" ultrastructure of a cell with a prominent chloroplast, nucleus and extracellular hydrocarbons (light intensity 450 μmol m^{-2} s^{-1} and nitrate concentration 0.1 mM). **b.** EPS-producing cell that was grown at 950 μmol m^{-2} s^{-1} and 6 mM nitrate. A severely reduced chloroplast and a prominent large vacuole with a system of membranes are readily visible. Hc – hydrocarbons, Chl – chloroplast, Nu – nucleus, Ps – polysaccharides, Sv – structured vacuole. Bar 5 μm.

an optimal combination of light and nitrogen concentrations, produce EPS in amounts comparable to high EPS-producing algal strains. *B. braunii* clearly belongs to a group of algae whose metabolism is governed by a complex of ambient conditions, resulting in the production of various metabolites of potential economic importance.

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