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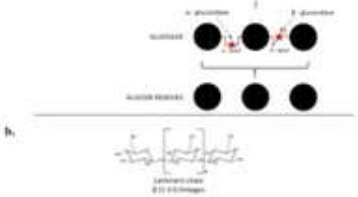

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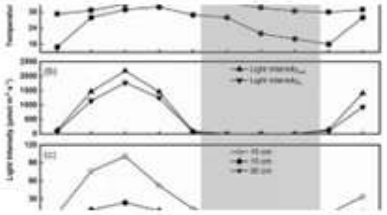

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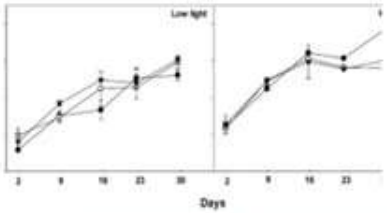

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Abstract

Botryococcus braunii CCAP 807/2 has been studied intensively for biofuel production due to its high hydrocarbon content. This strain is also capable of producing high value carotenoids. The aim of the study was to analyse the carotenoid production of *B.braunii* 807/2 under different growing conditions, first, by using different media and light intensities in indoors, and next, to examine the carotenoid composition between green, intermediately pigmented and red *B. braunii* grown in indoors and outdoors. The alga was cultured indoors under two different light intensities (100 and 500 µmol photons m^{-2} s^{-1}) using three different media: a control with complete modified CHU 13 medium, modified CHU 13 without N and modified CHU13 without N + 2Fe. All cultures were grown at 25 °C with 12:12 h light:dark cycle and were mixed with magnetic stirrers. For the determination of carotenoid composition at different stages, the green, intermediately pigmented and red cells were collected from indoor and outdoor cultures and analysed for their carotenoid composition using HPLC. The cultures grown at high light intensity reached the highest biomass yield at 0.6 g L^{-1} on day 16, whereas their counterparts at low light intensity took 30 days to reach the same biomass yield. The carotenoid production of *B. braunii* 807/2 at high light intensity increased up to twofold in 2 days compared to the ones grown at low light. *Botryococcus braunii* 807/2 accumulates lutein, canthaxanthin and astaxanthin and β,β-carotene as the main carotenoids. Whilst lutein was the major carotenoids of the green/intermediate cells, canthaxanthin and astaxanthin were the predominant carotenoids of the red cells under indoor and outdoor culture, respectively. This study suggests that *Botryococcus braunii* 807/2 is a potential candidate for the production of lutein and/or astaxanthin. It accumulates a high amount of lutein when grown under optimum conditions and a high amount of astaxanthin when grown under sub-optimum conditions outdoors.

Carotenoid production of *Botryococcus braunii* CCAP 807/2 under different growth condition

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Abstract

Botryococcus braunii CCAP 807/2 has been studied intensively for biofuels production due to its high hydrocarbon content. This strain is also capable of producing high value carotenoids. The aim of the study was to analyse the carotenoid production of *B.braunii* 807/2 under different growing conditions, first, by using different media and light intensities indoors, next, to examine the carotenoid composition between green, intermediately pigmented and red *B. braunii* grown in indoors and outdoors. The alga was cultured indoors under two different light intensities (100 and 500 μmol photons m^{-2} s^{-1}) using three different media: a control with complete modified CHU 13 medium, modified CHU 13 without N, and modified CHU13 without N + 2Fe. All cultures were grown at 25^{o}C with 12h:12h light:dark cycle and were mixed with magnetic stirrers. For determination of carotenoid composition at different stages, the green, intermediately pigmented and red cells were collected from indoor and outdoor cultures and analysed for their carotenoid composition using HPLC. The cultures grown at high light intensity reached the highest biomass yield at 0.6 g L^{-1} on day 16 whereas their counterparts at low light intensity took 30 days to reach the same biomass yield. The carotenoid production of *B. braunii* 807/2 at high light intensity increased up to two-fold in two days compared to the ones grown at low light. *Botryococcus braunii* 807/2 accumulates lutein, canthaxanthin and astaxanthin and β,β-carotene as the main carotenoids. Whilst lutein was the major carotenoids of the green/intermediate cells, canthaxanthin and astaxanthin were the predominant carotenoids of the red cells under indoor and outdoor culture, respectively. This study suggests that *Botryococcus braunii* 807/2 is a potential candidate for production of lutein and/or astaxanthin. It accumulates a high amount of lutein when grown under optimum conditions and a high amount of astaxanthin when grown under sub-optimum conditions outdoors.

Keywords : Astaxanthin; Chlorophyceae; light; Lutein; outdoor culture

1. Introduction

Carotenoids are lipid soluble pigments that give yellow, orange or red colours of, e.g., plant leaves, fruit, flowers, feathers, crustacean shells, flesh and skin of fish (Britton et al. 2004). They are produced de novo by all photosynthetic organisms and more than one hundred carotenoids are found in microalgae (Egeland 2016), but only a few are used and produced commercially, the two main ones from algae being β,β-carotene and astaxanthin (Borowitzka 2018). Carotenoids have an important role in the photosynthetic apparatus and also serve to protect cells from oxidative damage and high light (Solovchenko 2013; Hashimoto et al. 2016).

Considering the potential market value as well the range of potential applications of carotenoids especially β,β-carotene, astaxanthin, lutein and fucoxanthin, searching for new sources of carotenoids from microalgae with commercial potential is an important and challenging task. Several strains of the green alga *Botryococcus braunii* have been observed to turn orange to reddish in culture. The following carotenoids have been reported for *B. braunii*: lutein, β,β-carotene, β,ε-carotene, loroxanthin, neoxanthin, antheraxanthin, zeaxanthin, canthaxanthin and echinenone (Grung et al. 1989; Lee-Chang et al. 2020), adonixanthin and astaxanthin, with the latter two only found in stationary phase cells of an L-race strain (Grung et al. 1994c). The unusual carotenoids botryoxanthin A & B and braunixanthins 1 & 2 have also been reported for strains of the B-race of *B. braunii* (Okada et al. 1996; Okada et al. 1997; 1998). Echinenone, canthaxanthin and astaxanthin are clearly secondary carotenoids and echinenone and canthaxanthin have been found to be mainly localised in the extracellular matrix of the *B. braunii* colony (Grung et al. 1994b). One strain of *B. braunii* (BOT-20) is unusual in that is remains dark red in all stages of culture and the hexane/acetone extract consisted mainly of echinenone (Matsuura et al. 2012). Like many other green algae the cell carotenoid content increases in the stationary phase of growth as well as under increased salinity (Grung et al. 1994b; Ranga Rao et al. 2007) and echinenone content increases under high light (van den Berg et al. 2019). However, it is clear that the strains of *Botryococcus* not only vary in the type and amount of hydrocarbons they produce so that they have been classified into four 'races' (A,B,L,S) (Kawachi et al. 2012), but that they also vary in their carotenoid production. However, carotenogenesis and strain variation in carotenogenesis is still little studied in detail.

We observed that in the strain *B. braunii* CCAP 807/2 (race A) the colour of the culture changes from green to deep red/orange under certain conditions (i.e., high light, stationary phase). Some very old cultures (more than one year old) maintained in our lab exhibited bright red/orange colours. In addition, *B. braunii* 807/2 has been studied widely in relation to its hydrocarbon production including in outdoor culture (Moheimani et al. 2013b; Zhang 2013; Gouveia et al. 2017; Blifernez-Klassen et al. 2018; Kleinert and Griehl 2021). Hence, we have good understanding about the strain specifically related to its limits to growth factors for hydrocarbon production but not for carotenoid production. Therefore, the aim of this study was to analyse the carotenoids and the rate of carotenoid production of *B. braunii* 807/2 under different growth conditions.

2. Materials and Methods

2.1 The Source of the Strain

Botryococcus braunii CCAP 807/2 originally isolated by Jaworski in 1984 from Lake Grasmere, Cumbria, England (Hilton et al. 1988) was obtained from the Culture Collection of Algae and Protozoa (CCAP).

2.2 Effects of N-starvation, Fe and light intensity

Botryococcus braunii 807/2 was cultured in 1 L Schott bottles containing 750 mL of medium at two different light intensities (100 and 500 μmol photons m^{-2} s^{-1}) in three different media: a) control with complete modified CHU 13 medium (Chu 1942), b) modified CHU 13 without N, and c) modified CHU13 without N + 2Fe) (in triplicates). The nitrogen source used in modified CHU 13 was KNO_3 and the source of light used was LED lights. The inoculum was centrifuged and the supernatant was discarded before adding the fresh medium. All cultures were grown at 25ºC with 12h: 12h light:dark cycle and were mixed with magnetic stirrers. The experiment was run in batch mode for a month.

2.3 Carotenoid composition of green, intermediate and red cells (indoors & outdoors)

For indoor cultures, the green/intermediate green cells were collected from a 2 months culture grown at 25ºC, 150-200 μmol photons m^{-2} s^{-1} in a 20 L carboy containing about 15 L culture. The intermediate red and red cells grown at 25ºC, 150-200 μmol photons m^{-2} s^{-1} in several 1L flasks containing about 500 mL culture per flask were collected from cultures about 6 and 8 months old, respectively. The cells were collected by filtering the culture through Whatman GF/C filters.

For the outdoor cultures, the green/intermediate green cells of *B. braunii* collected from a 1 m^2 raceway pond (2-3 months old) were concentrated by first settling the cultures in a bucket, decanting the water and then filtering the cells through Whatman GF/C filters. The intermediate half green and half-red cells were collected from a 1 m^2 raceway pond (about 6 months old). To obtain the red cells, the intermediate half-green and half-red culture from the 1 m^2 raceway pond was transferred into a 10 m^2 raceway pond and then the pond was topped up with tap water only. The culture turned red after 1-2 weeks (note that this occurred in summer where solar irradiance ranged from 15-34 MJ m^{-2} s^{-1}). The red cells were harvested by scraping them off the wall of the pond as these cells stick to the pond's walls. The samples were then lyophilized and stored in a freezer until further analysis. The culture medium used for the indoor and outdoor culture was modified CHU 13. Carotenoid composition of green, intermediate and red cells for both indoor and outdoor samples were analysed by HPLC (High Performance Liquid Chromatography).

2.4 Analytical methods

2.4.1 Dry weight (DW) and ash-free dry weight (AFDW)

DW and AFDW were determined following the method of Moheimani et al. (2013a). Briefly, five mL of culture was filtered through pre-weighed and pre-combusted Whatman GF/C, 25 mm filter paper. The filters were then dried in an oven at 75°C for 5 hours. Dry weight (DW) was determined by the following equation:

$$\boldsymbol{Dry\ weight\ (g\ L^{-1}) = (weight\ of\ filters\ plus\ algae) - (weight\ of\ filters)}$$

The filters were then transferred to a furnace at 450°C and ashed for 5 h. Organic dry weight (Ash-free dry weight) was calculated by the following equation:

$$\boldsymbol{Ash-free\ dry\ weight\ (g\ L^{-1}) = (dry\ weight) - (weight\ after\ ashing)}$$

2.4.2 Lipid extraction

Lipid extraction was performed following the method of Bligh and Dyer (1959) as modified by Kates and Volcani (1966) and adapted by Mercz (1994). Briefly, 5 mL of the culture was filtered using Whatman GF/C filter paper. The filter containing cells was crushed with a glass rod to a paste and then extracted with 5.7 mL of a solvent mixed (methanol: chloroform: DI water in the ratio of 2:1:0.8 v/v/v). The extract was then centrifuged at 1107xg for 10 minutes. The supernatant was transferred into a 20 mL glass tube with screw cap. For the second extraction, 5.7 mL of solvent was added to the pellet and then vortexed and centrifuged. The supernatant was combined and added 3 mL of DI water and 3 mL of chloroform then vortexed before incubation in the refrigerator overnight for complete separation. After phase separation, the upper layer was removed carefully and several drops of toluene were added to the tube, shaken by hand and then transferred to a dry and pre-weighed 10 mL vial. The solvents were evaporated under a stream of pure N_2 gas on heating plates at 38°C until complete dryness. The vials containing lipids were then carefully weight using an analytical balance (Mettler Toledo AB135-S). Weight of lipids was calculated by subtracting the weight of vials containing lipids with the weight of the vials.

2.4.3 Chlorophyll determination

Chlorophyll concentrations were determined from acetone extracts using the method of (Jeffrey and Humphrey 1975). Briefly, 5 mL of the cultures was filtered through GF/C (Whatman) filter paper then folded and patted dry with paper towel. The cells were extracted with 4 mL of 90% cold acetone. The samples were then centrifuged for 10 minutes at 1107xg. The supernatant was transferred into a clean centrifuge tube. For the second extraction, about 2 mL of 90% of cold acetone was added the tube, vortexed and then centrifuged again at 1107xg for 10 minutes. The supernatant was combined giving the final extract volume of about 6 mL. The absorbance of the supernatant was measured at 664 and 647 nm.

The concentrations of chlorophylls *a* and *b* were calculated using the following equations for green algae (Jeffrey and Humphrey 1975):

$$\boldsymbol{Chlorophyll\ a\ (\mu g\ mL^{-1}) = 11.93\ A_{664}\ -\ 1.93\ A_{647}}$$

$$\boldsymbol{Chlorophyll\ b\ (\mu g\ mL^{-1}) = 20.36\ A_{647}\ -\ 5.50\ A_{664}}$$

2.4.4 Carotenoid analysis using quantitative Thin Layer Chromatography (TLC)

For TLC analysis, 5 mL of the cultures were filtered through GF/C (Whatman) filters. The filters containing cells were put in 4 mL glass test tubes and then crushed with a glass rod until a smooth red paste of about 0.5 mL was obtained. 1 mL of 90% ice-cold acetone was added into the tubes, homogenized well with a glass rod and then transferred in a plastic centrifuge tube with screw cap. Another 1 mL of acetone was added into the glass tube to wash and clean all the remaining cell debris which was also transferred into the centrifuge tubes and then centrifuged at 1107x*g* for 10 min. The supernatant was transferred to a 10 mL graduated glass centrifuge tube. For the second and third extraction, 2 mL of acetone were added to pellet in the centrifuge tube, vortexed to resuspend the pellets and then centrifuged again at 1107*xg* for 10 min and the supernatants were combined in the glass tube. The samples were reduced to about 2 mL under a stream of N_2. The same volume of petroleum spirit (boiling range point 40-60^{o}C) (about 2 mL) was added and then left for partition to occur. The upper phase was transferred into a vial and evaporated to complete dryness under a stream of N_2 gas. The dried pigment was stored in a freezer in a tightly sealed container under N_2 until further analysis. For thin layer chromatography the dried pigment was dissolved in 1 mL acetone. The pigment extract was applied/loaded as a 150 mm wide band to an activated silica gel plate (standard 20x20 cm aluminium-backed TLC plate/Merck TLC Silica Gel 60F254). Activation of the plate was done by heating the plate for 1 h at 120^{o}C. The loaded TLC plate was transferred into a saturated developing chamber containing 100 mL solvent mixture of acetone:hexane:petroleum spirit (boiling point range 40-60^{o}C) (20:10:70 mL; v/v/v). The pigment bands were identified based on the calculated R_f values and compared with R_f values in the literature. The pigment bands with yellow, red and orange colours were recovered by carefully scraping off using a plastic blade. Each individual pigment powder was eluted with 2 mL ethanol and then centrifuged at 1107*xg* for 10 min. The volume of the samples/supernatant was recorded and then the absorption spectrum was measured between 400-700 nm. Concentration of the carotenoids was calculated according to the following equation (Howieson 2001):

$$C = \frac{A \times 10}{E^{1\%}_{1\ cm}}$$

where:

C = concentration of carotenoid in mg mL^{-1}, A = measured absorbance at wavelength of maximum absorbance, $E^{1\%}_{1\ cm}$ = 2600 for β,β-carotene, $E^{1\%}_{1\ cm}$ = 2500 for xanthophylls (zeaxanthin, lutein, violaxanthin, neoxanthin) and $E^{1\%}_{1\ cm}$ = 2200 for keto-carotenoids (astaxanthin, canthaxanthin). The total carotenoid content was determined by summing all the

individual carotenoid concentrations recovered from TLC plate using UV-vis spectrometry. As a general precaution, all processes were done under dim light and the pigment extracts were kept cool at all times by placing them in a cool box on ice.

2.4.5 Carotenoid determination by HPLC

To obtain the best possible extraction solvent/procedure, several extraction methods were tested. All extractions were performed at room temperature in darkness (wrapped in a thick black cloth). A tip of a spatula with red or green cells was transferred into separate test tubes (test tubes with screw cap). Approximately 2 mL of solvent was added into the tube and then flushed with nitrogen gas to prevent oxidation. The following solvents or solvent mixtures were tested: A. 30 % methanol, 70 % acetone (Grung et al. 1994a; Grung et al. 1994b); B. benzene; C. methanol; D. ethanol; E. ethyl acetate; F. acetonitrile; G. acetone; H. 90 % acetone, 10 % water; I. isopropyl acetate; J. pyridine; K. chloroform; L. 50 % chloroform, 50 % methanol; M. hexane; N. the freeze dried cells were first moisturised by addition of a few drops of DI water and after 5 minutes approx. 2 mL of a mixture of 50 % chloroform, 50 % methanol was added.

Upon comparison of the extraction methods, treatments L and N showed the best results, although a bit of colour was still seen in the cells indicating that not all pigments were completely extracted from the cells. Treatment N was efficient for green cells. For red cells, treatment L was better. Because some samples contained a mixture of red and green cells, extraction method N was selected for the analysis of all samples. The lyophilised cells were soaked in solvent for three days at room temperature under nitrogen in darkness. After extraction, the cells were filtered and the residue flushed with a small amount of acetone followed by evaporation to dryness under reduced pressure with some absolute ethanol added before evaporation to remove the water. The dried pigments were immediately re-dissolved in acetone (approximately 1 mL) and analysed by HPLC. For all the above treatments, the cells with solvent were kept in a refrigerator, in darkness at approx. 2°C.

The HPLC separation was performed using an Agilent 1100 HPLC instrument with vacuum degasser, thermostatted autosampler with enlarged injection loop, quaternary pump, thermostatted column compartment, and diode array detector on two reversed phase C18 columns (ACE 5 C18 part no. ACE-121-2546, 4.6 x 250 mm each, with 5 mm packing) after each other, as described in Egeland (2012). The identity of the pigments was checked on a normal phase silica column (Grung et al. 1994a). All pigments were identified on the basis of retention time compared with an external standard and their visible spectra. Quantification was carried out on the basis of the calibration performed just before analyzing the samples. Pigments used for calibration were either donated or purchased from a commercial entity (CaroteNature, DHI, DSM, Sigma-Aldrich, and VWR), or isolated from a known source. The HPLC was calibrated for chlorophylls and carotenoids using reported absorption coefficients (Egeland 2011, 2012).

2.5. Statistical Analysis

Significant differences between treatments (control, CHU-N, CHU-N+2Fe) were analysed with a one-way analysis of variance (ANOVA) and Holm-Sidak and Tukey tests.

All statistical analyses were performed using Sigma-Plot 13 package (Systat Software Inc., USA).

3. Results

3.1 Effects of N-starvation, Fe and Light Intensity

The growth of *B. braunii* 807/2 was followed by determining its biomass (ash-free dry weight; AFDW) because it was difficult to follow growth based on the cell numbers or optical density as this strain was colonial. The alga grew very slowly. The biomass of the cultures grown at low light intensity (100 µmol photons m^{-2} s^{-1}) increased gradually from 0.35-0.4 g L^{-1} on day 2 to 0.55-0.6 g L^{-1} on day 30. On the other hand, the biomass of the cultures grown at high light (500 µmol photons m^{-2} s^{-1}) increased from 0.4 g L^{-1} on day 2 to a maximum of about 0.6 g L^{-1} on day 16 and entered stationary phase towards the end of the culture period, except for the control culture which continued to increase to around 0.7 g L^{-1} on day 30 (**Figure 1**).

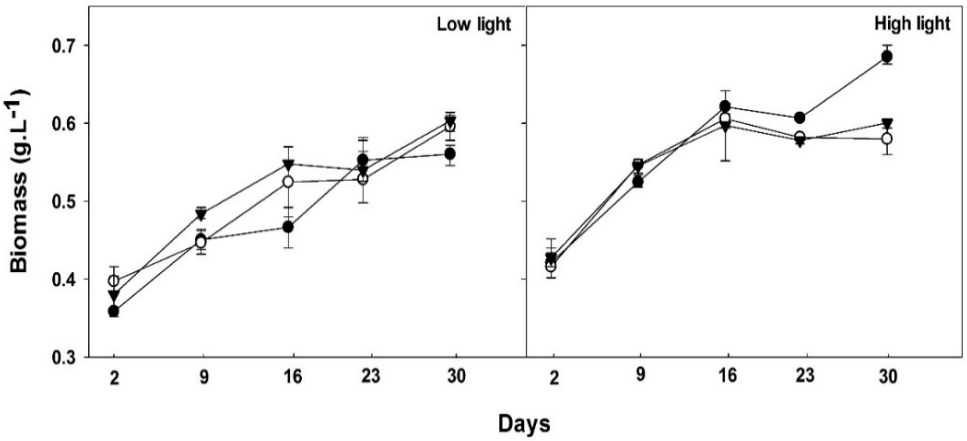

Figure 1. Biomass of *B. braunii* 807/2at low light (100 µmol photons m^{-2} s^{-1}) and high light (500 µmol photons m^{-2} s^{-1}) under different growing conditions (● = control, ○ = without nitrogen and ▼= without nitrogen+2Fe). Data represent mean±range, n=3

At low light intensity the lipid content showed a small fluctuation whereas at high light intensity there was a gradual increase in lipid over time. Based on the statistical analysis, there is no significant difference in the lipid content between treatments (One Way ANOVA, $p>0.050$). The average of the lipid content at low light was 0.1241±0.0211 g L^{-1} whereas the lipid content of their counterparts grown at high light was 0.1197±0.0236 g L^{-1} (Figure 2).

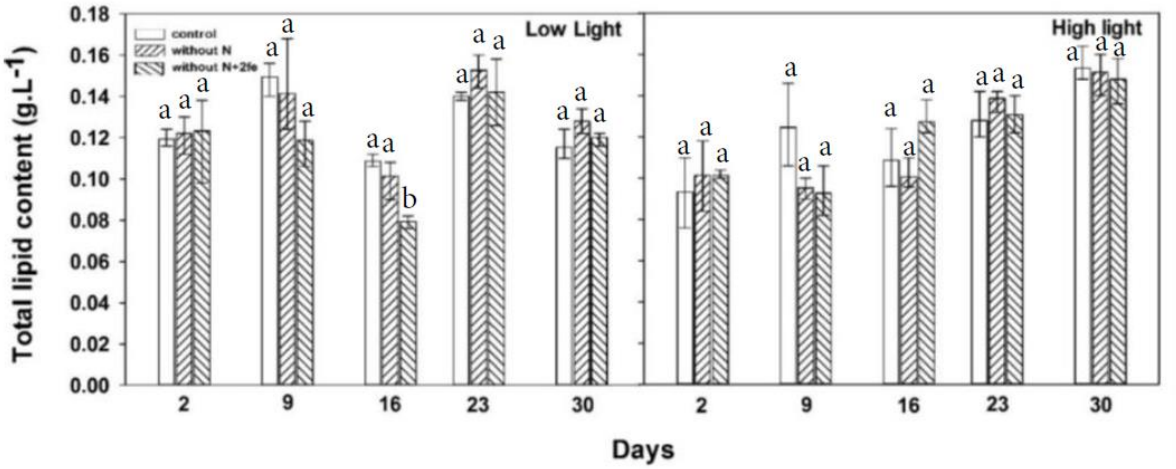

Figure 2. Total lipid yield of *B.braunii* 807/2 at low light (100 µmol photons m^{-2} s^{-1}) and high light (500 µmol photons m^{-2} s^{-1}) under different growth conditions. Data represent mean±range, n=3

The culture colour changed greatly with culture age, especially at high light intensity. The culture grown without nitrogen and with two times Fe changed markedly from green to yellow-orange colour on day 2 and stayed the same colour until about day 16 before changing colour to pale yellow. The culture grown without nitrogen also changed from green to yellowish colour on day 5, whereas the control culture remained green. At low light, control cultures remained green until the end of the culture period whereas the cultures grown under nitrogen limitation changed colour from green to brownish/yellowish after one week.

Chlorophyll *a* and *b* content of *B. braunii* grown under low and high light is shown in **Figure 3**. The chlorophyll *a* content of the control cultures under low and high light followed a similar upward trend reaching a maximum on day 23. The chlorophyll *a* content of the nitrogen limited cultures remained stable at around 4-6 mg L^{-1} under low light, whereas under high light, it decreased to below 2 mg L^{-1} from day 9. The chlorophyll *b* content of the control cultures under low and high light reached maximum on day 9 at around 3 mg L^{-1}. The chlorophyll *b* content of nitrogen limited cultures at low light remained steady at around 2 mg L^{-1} whereas their counterparts at high light decreased with time, reaching about 0.3 mg L^{-1} on day 30. Based on the statistical analysis, there is no significant difference in the chl *a* and *b* content between treatments in the first two days both at low and high light intensities (One Way ANOVA, $p>0.05$) but afterwards, significant differences in the chlorophyll content between treatments were observed (One Way ANOVA, $p<0.05$)

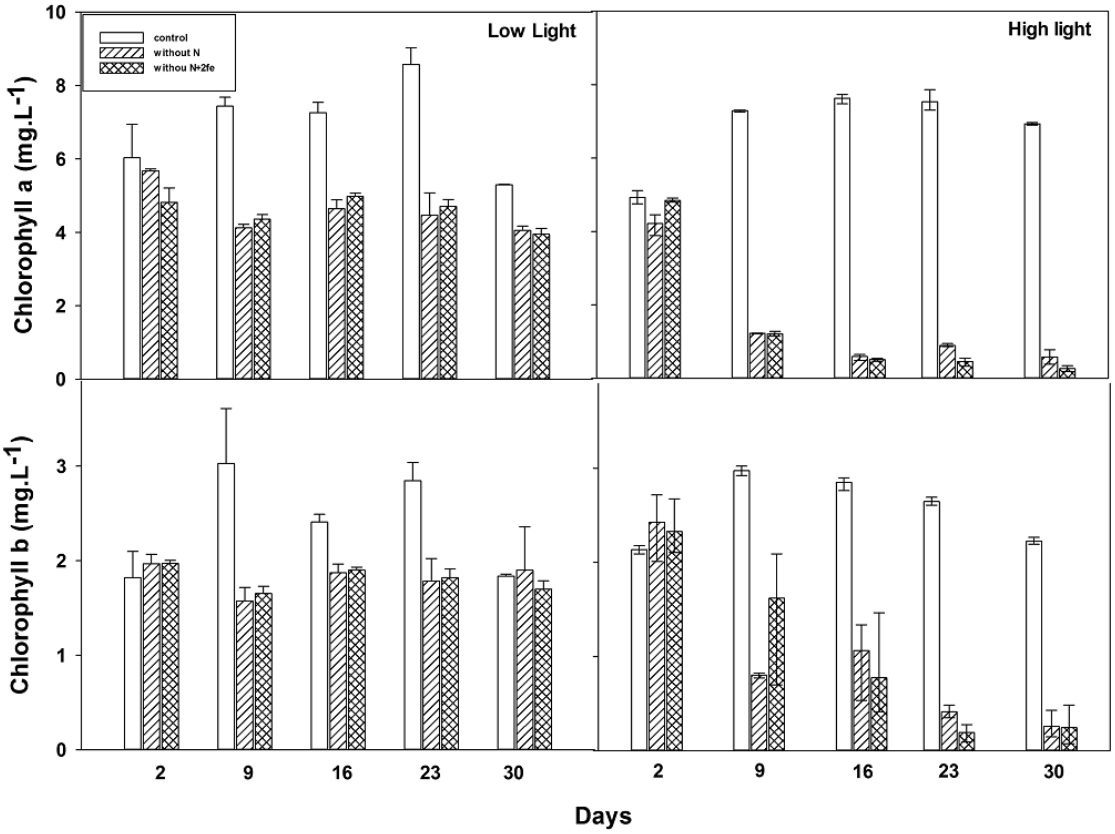

Figure 3. Chlorophyll *a* and *b* content of *B. braunii* 807/2 at low light (100 µmol photons m^{-2} s^{-1}) and high light (500 µmol photons m^{-2} s^{-1}) in three different media. Data represent mean±range, n=3

The carotenoid production of *B. braunii* 807/2 at high light was faster than that at low light (Fig 4). After two days of cultivation, the cultures grown under high light had 50-100% more carotenoids than their counterparts grown at low light **(Figure 4**). Irrespective of the growth conditions, the maximum carotenoid content at low light was achieved on day 9. At high light, the highest total carotenoid was also achieved on day 9 from the culture grown without N and with 2xFe concentration, whereas the control culture and the one without N had about 50% less carotenoid content than the one cultured with 2Fe concentration. Based on the biomass and carotenoid content, the carotenoid productivity and carotenoid content in % AFDW were calculated. The carotenoid content of *B. braunii* 807/2 ranged from 0.03-0.12% AFDW and the productivity of total carotenoid ranged from 0.01-0.04 mg L^{-1} day^{-1}.

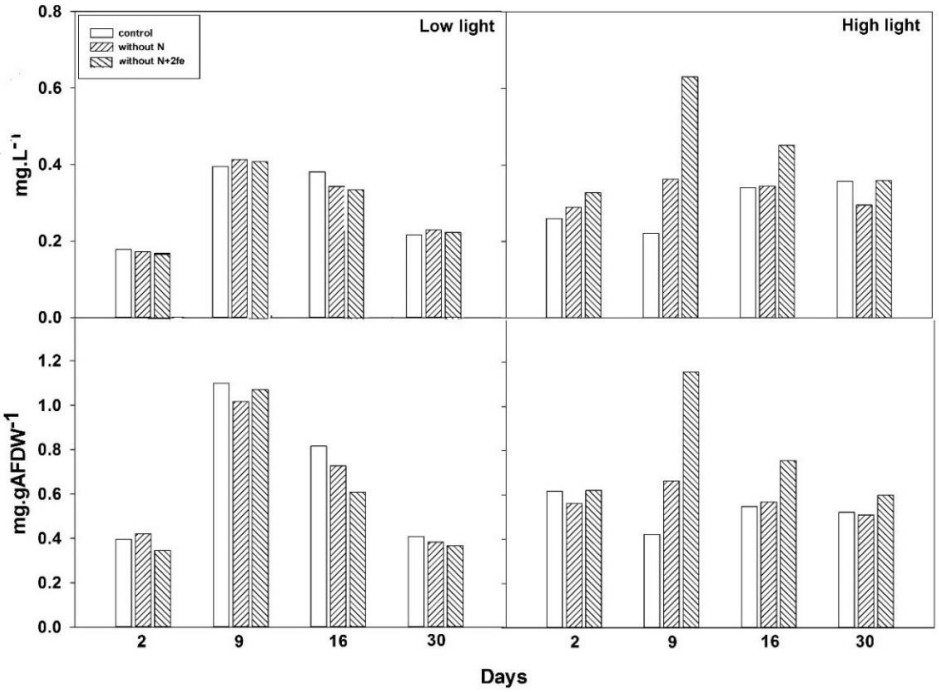

Figure 4. Total carotenoid content of *B. braunii* 807/2 at low light (100 µmol photons m^{-2} s^{-1}) and high light (500 µmol photons m^{-2} s^{-1}) in three different media (n=2)

At low light intensity the TLC plates showed several pigment bands including yellow pigments (β,β-carotene and lutein), olive green pigment (pheophytin), red/orange pigments (canthaxanthin and neoxanthin) and the prominent green pigments (chl *a* and *b*). At high light intensity, more yellow/red/orange pigments appeared as the culture aged with no more chlorophylls observed on day 16 from N-free medium and medium with extra iron.

3.2 Carotenoid composition of green, intermediate and red cells of *Botryococcus braunii* 807/2 cultured indoors and outdoors

The carotenoid composition of green, intermediate and red cells of indoor and outdoor cultures was compared to observe how the carotenoid composition changes over time. The green cells represented actively growing cells under optimal condition (i.e. nutrient sufficient). The intermediate cells were the cells that had started to change colour from green to red. These types of cells were mostly found at late stationary phase, whereas red cells were the cells that accumulated high amount of carotenoids accompanied by chlorophyll breakdown usually during stationary phase. The results for each pigment could not be given on a cell basis, only as % of total carotenoids, as even the best extraction methods did not result in complete extraction.

The results showed that *B. braunii* 807/2 accumulates lutein, canthaxanthin, astaxanthin and β,β-carotene as the main carotenoids. Whilst lutein was the major carotenoids of the green/intermediate cells, canthaxanthin and astaxanthin were the predominant carotenoids of the red cells under indoor and outdoor culture, respectively (**Figure 5**). As the cells change from the green to the red stage, major changes of some carotenoids were

observed. The most striking feature was the changes in lutein and astaxanthin content. The lutein content of intermediate cells decreased dramatically from 55% to 12% in the outdoor culture and from 43% to 5% in the indoor culture. In contrast, astaxanthin content increased significantly from being undetected in the green cells (both indoor and outdoor) to about 7% (indoor) and 28% (outdoor) (**Table1**).

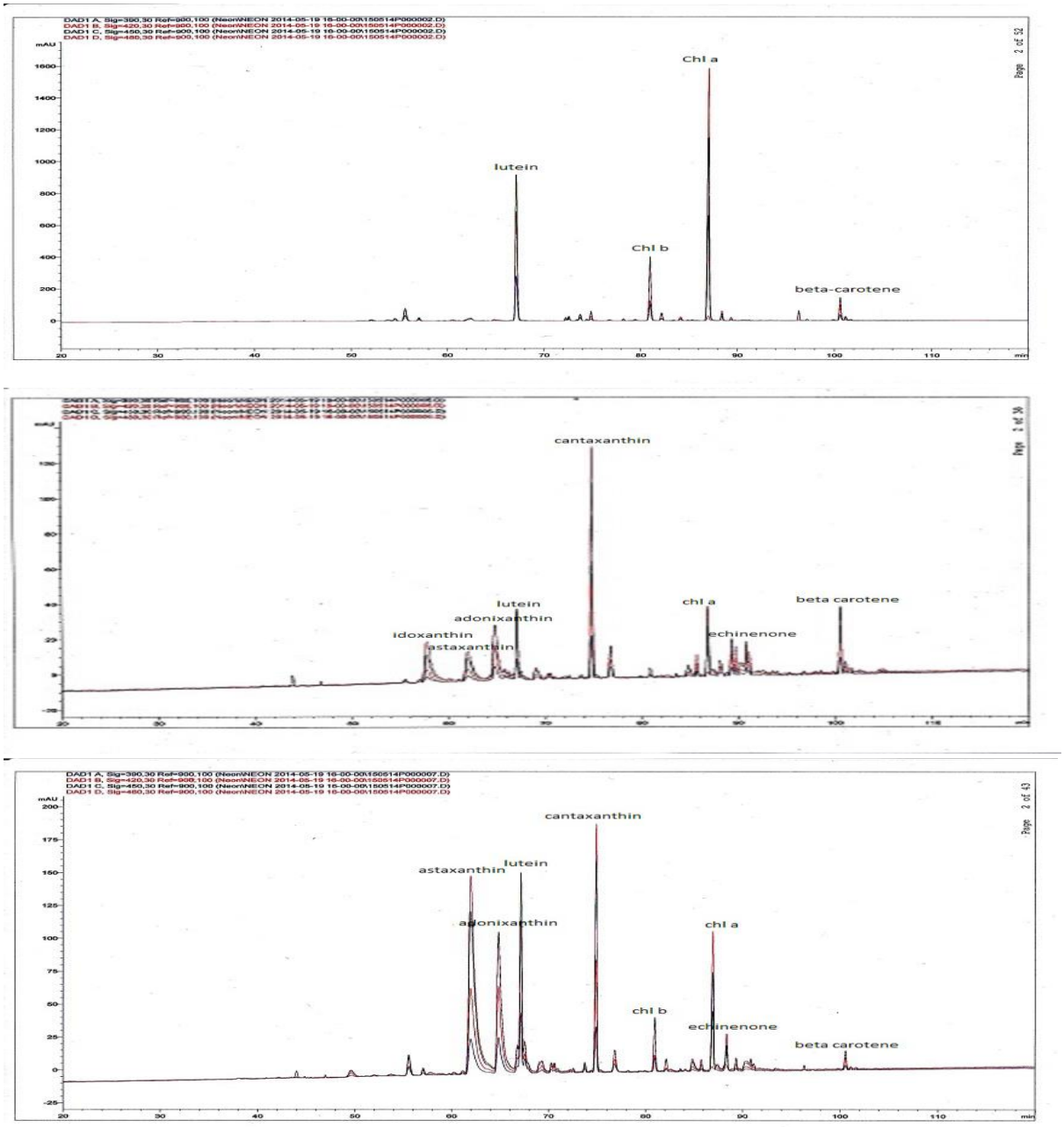

Figure 5. HPLC chromatograms of the green cells (top panel), the red cells (centre panel) cultured indoors, and the red cells from the outdoor culture (bottom panel)

Table 1. Carotenoid composition of green, intermediate and red cells of *B. braunii*807/2 grown indoors and outdoors as % of total carotenoids

Types of carotenoids	Green/ intermediate cells (outdoor)	Green/ intermediate green cells (indoor)	Intermediate half red and half green cells (outdoor)	Intermediate red cells (indoor)	Red cells (indoor)	Red cells (outdoor)
β,ε-Carotene	0.5	0.6	-	0.2	0.2	0.1
β,β-Carotene	7	8	2.1	2.9	4	0.9
Lutein	55	43	33	30	5	12
Violaxanthin	1.8	3.7	1.1	2.0	0.4	0.6
Neoxanthin (*cis*+*trans*)	10	9	5	2.1	0.5	2.3
Echinenone	1.2	1.7	0.7	2.1	9	0.7
Canthaxanthin	3.4	3.2	6	17	16	13
Adonixanthin	0.8	1.2	6	6	7	13
Astaxanthin	-	-	9	3.2	7	28
Idoxanthin	0.1	3.6	0.8	-	7	0.3
Total carotenoids with known identity (%)	79.8	74	63.7	65.5	56.1	70.9
Total of minor+degraded + unknown carotenoids (%)	20.2	26	36.3	34.5	43.9	29.1

Not all carotenoids could be identified. The total of minor, degraded and unknown carotenoids ranged from 20.2% (green cells indoors) to 43.9% (red cells indoors). The inability to accurately identify all the carotenoids was due limitation of available reference standards. For example, we did not have available botryoxanthins or braunixanthins as reference standards, so we could not indicate their presence in the algal cells. However, if they are present, they are only present in minor amounts. Some minor pigments could also be 3/3'/4'-hydroxyechinenone and adonirubin (metabolic intermediates in the biosynthesis of astaxanthin), as also here, no reference standard was available. Many of the minor carotenoids were assumed to be *cis* isomers, often present in extracts of lyophilised cells, despite not being present in intact cells.

4. Discussion

Botryococcus braunii is a slow growing alga (Wolf 1983; Wijihastuti et al. 2017; Gouveia et al. 2019). In the present study, it took about 16 days for the cultures to double their biomass under high light intensity and over 30 days under low light intensity in the indoor cultures. As suggested by Belcher (1968), the slow growth may be due to the colonial matrix of the alga that hinders gas exchange as well as the ability of the alga to direct metabolism into metabolically expensive lipids and hydrocarbons. Another possible explanation as suggested by Murray and Thomson (1977) is that the abundant matrix containing carotenoids and hydrocarbons reduced the light reaching the chloroplasts of the cells. Zhang (2013) studying the same strain of *B. braunii* 807/2 and found that the highest specific growth rate was about 0.45 day^{-1} between day 0 and 2 but afterwards, the specific

growth rate declined to an average of 0.04±0.03 day^{-1} or a doubling time of about 17 days. Growth rates appear to be strain-specific as shown by Li and Qin (2005) for *B. braunii* NIES-836 (0.09 day^{-1}), *B. braunii* UK 807/2 (0.18 day^{-1}) and *B. braunii* CHN 357 (0.2 day^{-1}).

Light is one of the most influential factors on growth of microalgae. In the current study, biomass production of the *B. braunii* was faster at higher irradiance. *B. braunii* prefers high light intensity for grow because naturally *B. braunii* floats on the surface of the water due to its matrix containing hydrocarbon so that it is exposed to high light intensity (Wake and Hillen 1980; van den Berg et al. 2019). This is in line with another study on *B. braunii* (Wang et al. 2019);

From this study it was found that the lipid content of the *B. braunii* CCAP 807/2 fluctuated. Similar conditions were also found by previous researchers using the same strain (Zhang 2013) and in different strains (Shokkumar and Rengasamy 2012). The possible explanation for this phenomenon is caused by the uniqueness of the algae *B. braunii* which releases its lipids into the medium. According to Suzuki et al (2013) the formation of lipids in the matrix occurs after cell division in the basolateral region of daughter cells. The size and inclusion of lipid bodies throughout the cell cycle change dynamically. Lipid body size and inclusions increase at the beginning of cell division until extracellular accumulation of lipids begins. Most of the lipids disappear from the cytoplasm along with extracellular accumulation and then reformation of the intra-cellular lipid take place (Suzuki et al. 2013).

The pigment content and composition of *B. braunii* 807/2 was examined under different growth conditions. The reason for testing N-free medium this is known to induce carotenogenesis in *Botryococcus*(Grung et al. 1994b) and other algae species such as *Haematococcus pluvialis* (Boussiba et al. 1999), *Scenedesmus* sp. (Pirastru et al. 2012), and other green algae (Orosa et al. 2000). Similarly, increased Fe^{2+} has been shown to enhance astaxanthin synthesis in *Chromochloris zofingiensis* (Wang et al. 2013) and *H. pluvialis* (Kobayashi et al. 1993; Choi et al. 2002), presumably by generating hydroxyl radicals (˙OH) through the Fenton reaction. Misawa et al. (1995) have also pointed out that Fe^{2+} is a cofactor involved in the catalysis performed by hydroxylases and oxidases required for astaxanthin synthesis.

In the present study, both high light and the -N + 2Fe treatment stimulated carotenoid production by day 9, although after this the total carotenoid content declined. This may be due to high light enhancing the oxidative effect of the hydroxy radicals followed by later acclimation of the alga to these conditions. Grung et al. (1994b) also found that carotenoid production of *B. braunii* is enhanced at high light intensity and nitrogen deficiency. Pirastru et al. (2012) found that *Scenedesmus* sp. accumulated high amounts of carotenoids, including astaxanthin and canthaxanthin, when exposed to low concentration of nitrogen, whereas *Chlorella zofingiensis* increased its astaxanthin levels at high irradiance (Del Campo et al. 2004). *B. braunii* 807/2 synthesized chlorophyll *a* and *b* as well as primary carotenoids (i.e., β,β-carotene, lutein, neoxanthin) under favourable conditions (nutrient sufficient and low light). However, under unfavourable condition (nutrient limitation, extra iron or high light intensity), the strain accumulated more secondary carotenoids, e.g., canthaxanthin and astaxanthin, concomitant with reduced chlorophylls and primary carotenoids.

The total carotenoid content of the *B. braunii* 807/2 presented in this study was relatively low (0.17-0.6 mg L^{-1} or 0.3-1.2 mg g^{-1}AFDW or 0.03-0.12% AFDW) compared to other studies on carotenoid content of *B. braunii*. For example, Grung et al. (1994c) reported

a total carotenoid content of 0.23% of DW in red cells of *B. braunii* YIC2, a L-race strain. Ranga Rao et al. (2010) reported a total carotenoid content of 0.35±0.6% of DW (predominantly as lutein) in *B. braunii* CFTRI-K grown at a low irradiance of approx. 20.5 µmol photons m^{-2} s^{-1} for 4 weeks; the total carotenoid content was determined by spectrophotometry using the Lichtenthaler (1987) equation which is likely an over estimation. In the present study, the total carotenoid content was determined from chlorophyll-free samples by determining carotenoid content of each individual carotenoid recovered from TLC plates using UV-vis spectrophotometry (see Materials & Methods) and then summing them to obtain total carotenoid content. This method has the potential for loss of pigment during the process and thus is likely an underestimation. The different extraction methods are also likely to have some effect on the results.

The carotenoid composition of *B. braunii* 807/2 is comparable to the results of previous studies of *B. braunii* strains in terms of the main carotenoids under optimum growing conditions, i.e., low light and nutrient sufficiency in which lutein is the predominant carotenoid of the green cells. For example, in *B. braunii* Kawaguchi-1 lutein comprised 76.2% of the total carotenoids (Tonegawa et al. 1998). Ambati et al. (2010) also found that lutein is the major carotenoid of *B. braunii* (79-84%). Similarly, in two *B. braunii* race B and race L strains lutein was the major carotenoid in the linear phase of growth (Grung et al. 1989).

The red cells of *B. braunii* 807/2 contained the keto-carotenoids canthaxanthin and astaxanthin as major pigments. The bright red colouration of the cells was due to a masking of the chlorophylls by the astaxanthin/canthaxanthin accumulation or may in part due to the reduced chlorophyll content after long exposure to high light intensity under N-limitation. Interestingly, there was a major difference in the carotenoids between the indoor and outdoor red cultures. Compared to other *B. braunii* strains (Grung et al. 1989; Tonegawa et al. 1998; Matsuura et al. 2012), the carotenoid composition of *B. braunii* 807/2 is unique since astaxanthin is the major carotenoid (28% of total carotenoids) of the red cells grown outdoors, whereas the red cells grown indoors contained predominantly canthaxanthin (16% of total carotenoids). Echinenone is high in the red cells of the indoor culture, but low in the red cells of the outdoor culture. The only other report of astaxanthin in *B. braunii* is that of in *B. braunii* YIC2 where it comprised 5% of the total carotenoids. It appears that the much higher irradiance outdoors stimulates massive accumulation of astaxanthin. Whether other strains of *B. braunii* produce astaxanthin under very high light is unknown as all other studies have been carried out under lower irradiances indoors. The fact that *Botryococcus braunii* 807/2 accumulate these pigments under different culture conditions means the production of either carotenoid can be achieved through the management of culture conditions.

5. Conclusion

Lutein and astaxanthin are pigments of commercial interest (Saini and Keum 2018) and *Botryococcus* has been suggested as a potential source of carotenoids (Ambati et al. 2018). *Botryococcus braunii* CCAP 807/2 can be considered as a potential candidate for the production of lutein and/or astaxanthin. It showed its capability to accumulate high amount of carotenoids under nitrogen limitation, high iron concentration and high light intensity. It accumulates a high amount of lutein when grown under optimum conditions and a relatively

high amount of astaxanthin when grown under sub-optimum conditions (i.e. nutrient deficiency and high light intensity outdoors). Most importantly, *B. braunii* can well be cultured outdoors.

Further studies are needed to optimise the production of the potential carotenoids (i.e. lutein and astaxanthin) including determination of the best growing conditions (indoor and outdoor), the best harvesting time and the best extraction method for optimum production of the carotenoids. The possibility of co-production of carotenoids and hydrocarbons, especially with hydrocarbon production by 'milking' (Moheimani et al. 2014) is worth exploring, i.e. the 'old' biomass after several round of 'milking' may be a valuable source of carotenoids. However, it is important to recognise that the content and productivity of these carotenoids are low when compared to other algae such as *H. pluvialis* (Cysewski and Lorenz 2004) and *Scenedesmus almeriensis* (Fernández-Sevilla et al. 2010). Furthermore, the carotenoids are extracted as a complex mixture with other carotenoids such as canthaxanthin and echinenone which complicates marketing of the product, although it may be suitable as a pigmenter in animal feeds for poultry or fish.

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Data availability: Data are available for the authors upon reasonable request.

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