1 Title

2 Effects of 2-azahypoxanthine on extracellular terpene accumulations by the green microalga

3 *Botryococcusbraunii*, race B

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15 Article Info

16 Keywords:

17 *Botryococcusbraunii*, Hydrocarbon, Botyrococcene, Secondary carotenoid, 2-azahypoxanthine

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19 Abbreviations:

AHX, 2-azahypoxanthine; TMS, tetramethylsqualene; ECM, extracellular matrix; AOH, 2-aza-8-oxohypoxanthine; GC-EIMS, gas chromatography-electron impact mass spectrometry; HPLC, high performance liquid chromatography; LC-APPIMS, liquid chromatography-atmospheric pressure photoionization mass spectrometry

Abstract

The B race of the green microalga *Botryococcus braunii* accumulates large amounts of triterpene hydrocarbons that are attractive as a source for biofuels. The alga, however, exhibits rather slow growth and this characteristic has hindered its practical application for biofuel production. In order to test a phytohormone-like compound that may regulate the growth or hydrocarbon production of this alga, the effects of 2-azahypoxanthine (AHX) on algal biomass and terpene contents in the extracellular matrix (ECM) in the Showa strain of *B. braunii* were investigated. The biomass of the Showa strain increased with AHX supplementation under static culture conditions although such effects were not observed in cultures with aeration or agitation. AHX supplementation significantly induced the accumulation of secondary carotenoids, including species-specific tetramethylsqualene (TMS)-conjugated carotenoids, botryoxanthin A and braunioxanthin 1. Conversely, the addition of AHX showed a tendency to slightly lower the triterpene hydrocarbon (botryococcenes) content in the ECM. These metabolic changes in tri- and tetraterpene accumulation could be caused by a reduction in nitrogen uptake from the culture medium due to AHX addition. Thus, there is a possibility that AHX could be used as a reagent to modulate terpene biosynthesis in the B race of *B. braunii*.

1. Introduction

The mass consumption of fossil fuels has caused unprecedented atmospheric concentrations of greenhouse gases such as carbon dioxide, which results in ongoing global warming and eventually leads to acidification of the aquatic environment. The possible impact on climate, the aquatic environment, and other ecosystems is expected to be significant and on a global scale, thus the current situation has urged the development of alternative renewable energy sources, including biofuel from microalgae [1].

Among the possible biofuel sources, the colony forming green microalga *Botryococcus braunii* Kützinger, which belongs to the Trebouxiophyceae class [2] has attracted considerable attention owing to its ability to accumulate large amounts of hydrocarbons in its extracellular matrix (ECM). *B. braunii* can be classified into at least three chemical races – A, B, and L [3–6] – based on the types of hydrocarbons produced. Among these hydrocarbons, branched triterpene hydrocarbons produced by the B race, such as botryococcenes and methylsqualenes, are touted as potential jet fuels [7,8]. Despite the considerable potential of the alga as a biofuel source [9], its slow growth and the poor understanding of the regulation of this alga's metabolism have hampered its practical use. The B race of *B. braunii* is also known to accumulate secondary carotenoids [10], including species-specific tetramethylsqualene (TMS)-conjugated derivatives, botryoxanthins and braunioxanthins, in its ECM [11][12] (Fig. 1). Although triterpene-derived botryococcenes and tetraterpene-derived carotenoids share the common terpene biosynthetic precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) exclusively derived from the methylerythritol 4-phosphate (MEP) pathway [13–15] (Fig. 2), their metabolism is believed to be differentially regulated. For example, the highest activity of enzymes involved in botryococcene biosynthesis are mainly observed during periods of active cell division [16]. In this context, the B race of *B. braunii* does not accumulate botryococcenes under nitrogen deficient

71 conditions during which most oleaginous microalgae start to accumulate triacylglycerides [17]. On
72 the other hand, secondary carotenoid amounts increased during the stationary phase or under nitrogen
73 deficient conditions in the alga [10,16,18,19]. Thus, it seems that tri- and tetraterpene accumulations
74 are inversely coordinated with algal growth stages in *B. braunii*, although the underlying
75 mechanisms for this phenomenon is still largely unknown. To understand such regulatory
76 mechanisms for terpene biosynthesis in this alga, phytohormone-like compounds that affect growth
77 or terpene production could be good experimental tools.

78 A recently discovered bioactive natural compound, 2-azahypoxanthine (AHX) has garnered
79 considerable research interest as it has an ability to stimulate growth of crop plants and increase their
80 yields [20–22]. It was originally identified from a fungus, *Lepistasordida*, as the compound
81 responsible for the formation of “Fairy rings”, the phenomenon in which a ring of fungi causes
82 nearby plants to grow rapidly [20]. In a more recent study, AHX and its oxidized derivative, 2-aza-8-
83 oxohypoxanthine (AOH), were also isolated from diverse plant species including green algae [23].
84 It was proposed that AHX is synthesized *in vivo* from the intermediate of the purine metabolism
85 pathway, 5-aminoimidazole-4-carboxamide (AICA) [23], and subsequently oxidized to AOH,
86 suggesting that AHX and its metabolites are widely conserved bioactive compounds among
87 plants (Fig. 3). Although the overall picture of this signaling pathway at the molecular level is still to
88 be elucidated, its diverse effects on higher plants, from affecting ammonium uptake ability in root
89 of rice [20] to stimulating carotenoid accumulation in juice sacs of citrus, have already been
90 described [24].

91 The recent discovery of the diverse functionality of AHX in higher plants therefore
92 prompted us to investigate the effect of AHX on the growth and terpene metabolism of *B.*
93 *braunii* race B, strain Showa [25] and several findings are described herein.

Fig. 1. Planar structures of representative secondary carotenoids identified in the B race of *B. raunii*. (a) β -Echinenone, (b) **1**: braunixanthin 1, **2**: braunixanthin 2, (c) **1**: botryoxanthin A, **2**: botryoxanthin B, **3**: α -botryoxanthin A.

Fig. 2. Schematic diagram of tri- and tetraterpene biosyntheses in the B race of *B. braunii*. MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranyl-geranyl pyrophosphate; PSPP, presqualene pyrophosphate; SQS, squalene synthase; SSL1, squalene synthase-like protein 1; SSL2, squalene synthase-like protein 2; SSL3, squalene synthase-like protein 3 [27].

Fig. 3. Structure of AHX and its proposed biosynthetic pathway [23].

2. Materials and methods

2.1. Reagents

AHX was synthesized as previously described[26]. Authentic standards of C₃₀botryococcene from genetically modified yeast [27] and braunixanthin 1 from the Showa strain of *B. braunii*[12] were used for this study. All other reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Strain culture

In-house maintained *B. braunii* Showa strain[25] was used in the current study.

Cultures were grown in modified Chu13 medium [10] at 25°C with a 12:12 light/dark cycle at a light intensity of 120 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ from white fluorescent lamps. Three different culture experiments were conducted in relation to the physical agitation of the culture media. Static cultures were carried out with no aeration or no constant mechanical agitation but manual shaking of the culture vessels were conducted once per day. Cultures agitated by aeration were done by bubbling 2% CO_2 -enriched sterile air. For cultures agitated by mechanical shaking, culture flasks were continuously shaken on a Double Shaker NR-30 (TAITEC) at 100 rpm.

The composition of the basal modified Chu13 medium was as follows: KNO_3 800 $\text{mg}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 100 $\text{mg}\cdot\text{L}^{-1}$, $\text{K}_2\text{HPO}_4\cdot 3\text{H}_2\text{O}$ 52 $\text{mg}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 54 $\text{mg}\cdot\text{L}^{-1}$, FeNaEDTA 10 $\text{mg}\cdot\text{L}^{-1}$, H_3BO_3 2.86 $\text{mg}\cdot\text{L}^{-1}$, $\text{MnSO}_4\cdot \text{H}_2\text{O}$ 1.54 $\text{mg}\cdot\text{L}^{-1}$, $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.22 $\text{mg}\cdot\text{L}^{-1}$, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ 0.08 $\text{mg}\cdot\text{L}^{-1}$, $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ 0.06 $\text{mg}\cdot\text{L}^{-1}$, and $\text{CoSO}_4\cdot 7\text{H}_2\text{O}$ 0.09 $\text{mg}\cdot\text{L}^{-1}$. For culture experiments with mechanical agitation, the concentration of KNO_3 was reduced to 400 $\text{mg}\cdot\text{L}^{-1}$. AHX was added to a final concentration of 1 mM. Culture media were sterilized by filtration through a 0.45 μm membrane after pH was adjusted with H_2SO_4 or KOH to 7.5.

2.3. Biomass evaluation

Aliquots of algal cultures were harvested by vacuum filtration with pre-weighed GF/C glass fiber filters (Whatman) at weekly intervals. After washing three times with deionized water, the filters were freeze-dried to a constant weight and the biomass of samples was evaluated gravimetrically.

2.4. Determination of total secondary carotenoids in the ECM

Freeze-dried algal samples harvested by filtration with 10 μm mesh nylon filters or Whatman GF/C glass fiber filters were extracted by soaking into ca. 5 times volume of *n*-hexane for

10 min. Twenty micrograms of *n*-hexadecane was added as an internal standard to the samples before extraction to calculate the recovery ratio for normalization, as described in the following section. Extraction was repeated three times and each extract was filtered through a cotton plug, combined, and evaporated to dryness. The resulting residue was dissolved in 5 mL *n*-hexane and total secondary carotenoid content was determined colorimetrically with a BioSpec-1600 Spectrometer (Shimadzu), using the extinction coefficient of $E_{1\text{cm}}^{1\%} = 2500$ at λ_{max} [28].

2.5. Analysis of secondary carotenoid composition in the ECM

An aliquot of four milliliters was taken from the extract used for the determination of total secondary carotenoids, concentrated under reduced pressure, and subjected to silica gel open column chromatography (Wakogel C-300, Wako, 10 mm·7 mm i.d.). Initially, the colorless hydrocarbon fraction was separated by eluting with 5 column volumes of *n*-hexane. The remaining secondary carotenoid fraction was then eluted with 2 column volumes of acetone.

The recovered total secondary carotenoid fraction was further subjected to ODS open column chromatography (YMC ODS-AM, YMC, 50 mm·22 mm i.d.) to separate conventional C₄₀ carotenoids from TMS-conjugated carotenoids. The fraction containing conventional C₄₀ carotenoids was eluted using 5 column volumes of MeCN/CH₂Cl₂/MeOH (1:2:7) as the initial mobile phase. The retained fraction containing TMS-conjugated carotenoids fraction was then recovered by eluting with 2 column volumes of CH₂Cl₂.

The TMS-conjugated carotenoid fraction was further subjected to silica gel open column chromatography (Wakogel C-300, Wako, 10 mm·7 mm i.d.). The botryoxanthin fraction was eluted by 5 column volumes of *n*-hexane/benzene (2:1), followed by elution of the braunianthin fraction by 2 column volumes of acetone. Each of the three fractions (conventional C₄₀ carotenoid, botryoxanthin, and braunianthin fractions) was evaporated to dryness and dissolved in 5 mL of *n*-

hexane, and the carotenoid content was measured colorimetrically as described in the previous section.

Components in the fractions containing C_{40} carotenoids, botryoxanthin, and braunianxanthin were identified by LC-APPIMS system equipped with DGU-20A3 degasser, SIL-20AC injector, LC-20AD pump, CTO-20AC column oven, SPD-M20A photo-diode array detector (Shimadzu), and Amazon SL (Bruker) mass spectrometer. Fifty microliters of each fraction was injected into a Cosmosil 5C18 MS-II column (250 mm $\times$ 10 mm i.d., Nacal Tesque) and eluted at a flow rate of 1 mL $\cdot$ min⁻¹. Individual compounds were detected at wavelengths from 300 to 700 nm. Column oven temperature was 25 °C, capillary voltage was 4500 V, and endplate offset voltage was 500 V. For C_{40} carotenoid fraction, two mobile phases were used: mobile phase (A), H₂O/MeCN 1:9 and (B), EtOAc. The program was initiated with the concentration of (B) at 10 % that was increased up to 70% in 20 min, and kept at 70% for 10 min. The column was equilibrated for 10 min between analyses with the initial mobile phase.

For botryoxanthin or braunianxanthin fraction analyses, the following mobile phases were used: mobile phase (A), MeCN and (B), acetone. The program was initiated at 50% of (B), kept at 50% for 5 min, increased from 50% to 100% (B) in 15 min, and kept at 100% (B) for 10 min. The column was equilibrated for 10 min between analyses with the initial mobile phase.

2.6. Hydrocarbon analysis

The remaining 1 mL aliquots of extracts for total carotenoid determination were further used for hydrocarbon analyses by GC-EIMS. Hydrocarbon analyses were carried out on a GCMS-QP2010 Ultra (Shimadzu) equipped with an InertCap 1MS column (GL Science, df 0.25 μ m, 60 m, 0.25 mm i.d.) under the following conditions; carrier gas He; flow rate 2.58 mL $\cdot$ min⁻¹; vaporizing chamber temperature 250 °C; ion source temperature 250 °C; ionization voltage 70 eV. Column

temperature was held at 50°C for 1 min, raised to 220°C at 10°C · min⁻¹, then to 260°C at 4 C · min⁻¹, and finally held at 260°C for 22 min. Chromatograms were obtained in the total ion current (TIC) mode. Peaks displaying specific mass fragmentation patterns to terpene-derived compounds and retention times between those of C₃₀ botryococcene and squalene standards were identified as botryococcene homologues. Carbon numbers for individual molecular species of botryococcenes were assigned based on their molecular ion peaks. The amounts of hydrocarbons were quantified by peak areas derived from TIC using a calibration curve obtained from a dilution series of squalene as an external standard. The peak area of *n*-hexadecane added to each sample as an internal standard was utilized to normalize the recovery ratio.

2.7. Measurement of residual nitrate content in culture media

Algal cultures which were dedicated for the shaken culture experiment shown in Fig. 5b were used for measurement of residual nitrate content in culture media. Algal cultures were centrifuged at 400 g at 4°C for 10 min to pellet the algal cells. The resulting supernatant was subjected to nitrate content measurement using an anion selective electrode (LAQUATwin, HORIBA). Measurement of nitrate concentration was unaffected by dissolved AHX itself.

3. Results and discussion

3.1. Effect of AHX on biomass of *B. braunii*

In static cultures, AHX treatment at a final concentration of 1 mM resulted in a significant increase in the biomass of *B. braunii* (Fig. 4a), by up to 38% compared to the control after 4 weeks of culture. This effect on biomass was observed after 2 weeks of culture and remained consistent throughout the duration of the experimental period. The addition of the structural analogue of AHX, hypoxanthine, at the same concentration, on the other hand, completely inhibited algal growth (Sup.

Fig. 1a) and caused chlorosis of the algae (data not shown), indicating that the effects observed under AHX supplementation were specific to this compound. Quite intriguingly, more algal colonies were observed floating at the surface of the culture medium in static cultures supplemented with AHX (Fig. 4c right) than in control cultures with Chu13 medium (Fig. 4c left). This floating phenomenon could not be simply explained by differences in specific gravities between the two culture media because the Chu13 medium of which the concentration was raised to 130% in order to make its specific gravity similar to that of the medium containing 1 mM of AHX did not significantly cause floating of algae (Fig. 4c middle). Morphologies of algal colonies treated with AHX did not show any significant difference from that of control cells without AHX (data not shown). The mechanism of this floating phenomenon has still not been clarified. On the other hand, AHX treatment did not significantly increase biomass in aerated cultures despite the enrichment of the air for aeration with 2% CO₂ to enhance algal growth (Fig. 4b). In fact, the obtained biomass for AHX-treated cultures was slightly lower by ca 0.5 mg/10 mL (6% decrease) than the control group at 2 weeks after inoculation. These results raised two hypotheses; (i) AHX increased algal biomass in static cultures by giving more access to light or atmospheric air, because AHX-treated algal colonies were observed to be more buoyant and aggregated more at the surface of the medium compared to control cultures, (ii) the effect of AHX on algal growth was only exhibited under low CO₂ concentrations, *e.g.* it enhances CO₂ affinity under CO₂ limiting conditions.

To test this, comparison between static cultures and constantly shaken cultures without CO₂ supplementation was performed. In this set of experiments, the positive effect of AHX on algal biomass was also not observed in the constantly agitated cultures, supporting the initial hypothesis that it was the increased buoyancy and surface aggregation of AHX-treated cultures that contributed to the increase in biomass in static cultures (Fig. 5a and b). Currently, the underlying

mechanism(s) for the enhanced buoyancy of *B. braunii* colonies under AHX supplementation in static cultures has not been uncovered and will be an interesting focus for further research in the future.

Fig. 4. Effects of AHX on the biomass of *B. braunii*. (a) Change of algal biomass in static culture with AHX. (b) Change of algal biomass in aerated culture with AHX. ($n = 3-5$, data is represented as mean $\pm$ S.E.M., **: $P < 0.01$, Student's t -test.) (c) Appearance of *B. braunii* in static cultures. Left to right, Control: Chu13 medium, 130%: 130% concentrated Chu13 medium, AHX: Chu13 medium + 1mM AHX. A red arrow indicates that algal body was floating on the surface of the culture medium. The photograph was taken 3 days after inoculation. A representative image was taken from two independent experiments.

Fig. 5. Comparison of algal growth between (a) static and (b) shaken culture conditions. No CO_2 was supplemented under both culture conditions. ($n = 3$, data is represented as mean $\pm$ S.E.M., *: $P < 0.05$, **: $P < 0.01$, Student's t -test).

3.2. Effect of AHX on extracellular botryococcene content of *B. braunii*

GC-EIMS analysis of algal extracts indicated the presence of 8 major botryococcenes in the Showa strain (Fig. 6a, Table 1). The hydrocarbon composition was similar to those reported in previous studies for the same strain [29,30]. Despite this similarity, the relative amount of botryococcenes (hydrocarbon content/dry weight %) in algae showed a tendency to be lower in AHX-treated cultures at the end of the culture period under both static and aerated culture conditions (9.5% decrease in static and 6.6% decrease in aerated culture, Fig. 6b, c). In static culture treated with AHX however, the increase of total algal biomass during 4 weeks was sufficiently large (Fig. 4b) to compensate for the reduction of relative hydrocarbon contents per algal dry weight (Fig. 6b). Thus, in terms of the total amount of hydrocarbons produced, AHX-treated algae produced more botryococcenes than control cultures during the same period of static culture cultures by 10% after 4 weeks of culture (Fig. 5d). In contrast, the total amount of botryococcenes produced in aerated cultures were lower in AHX-treated algae than those cultured with Chu13 by 25% because both algal biomass and relative hydrocarbon content per algal dry weight were reduced under AHX supplementation (Fig. 6e). The decrement of total hydrocarbons (ca. 0.7 mg/10 mL culture in Fig. 6e) in the AHX-treated algae at two weeks after inoculation was almost equivalent to that of the reduction in biomass (ca. 0.5 mg/10 mL culture in Fig. 6b). This indicates that AHX had little effect on the production of any other components in the algal biomass other than hydrocarbons.

In this context, C₃₀ botryococcene is known to be sequentially methylated to yield botryococcene homologues with higher carbon numbers [19,31]. When *de novo* synthesis of C₃₀ botryococcene is very active and methylation cannot catch up with it, the ratio of less methylated botryococcene homologues so called “lower” botryococcenes from C₃₀-C₃₂ to “higher” botryococcenes from C₃₃-C₃₄ becomes high [19,32]. While the amount of botryococcenes was decreased in AHX-treated cultures, the ratio of a lower (C₃₂) to higher (> C₃₂) botryococcenes

remained almost unchanged in algal samples cultured with aeration for 2 weeks (Table 1, Fig. 6f), indicating that the balance between *de novo* synthesis of C₃₀botryococcene and methylation was not affected by AHX treatment.

Therefore, AHX seemed to be concertedly reducing both *de novo* synthesis of C₃₀botryococcene and subsequent methylations.

Table 1. Relative composition of botryococcenes in the Showa strain cultured with or without AHX. Different alphabet denotes different homologues. Data is shown as mean±S.E.M (*n* =3). Algal samples cultured with CO₂ supplementation for 2 weeks were used.

Peak number	Molecular species	Relative retention time (squalene = 1)	Control	AHX
1	C ₃₄ a	0.904	8.7±0.1	7.1±0.1
2	C ₃₂ a	0.911	2.3±0.1	2.3±0.1
3	C ₃₄ b	0.922	63.5±1.0	55.0±1.0
4	C ₃₄ c	0.927	5.2±0.4	4.7±0.7
5	C ₃₄ d	0.935	5.0±0.1	4.3±0.2
6	C ₃₄ e	0.941	5.0±0.2	12.6±0.0
7	C ₃₃	0.947	9.2±0.1	12.4±0.1
8	C ₃₂ b	0.952	1.1±0.2	1.5±0.1

Fig. 6. Effect of AHX on botryococcene content. (a) A representative GC-EIMS total ion current chromatogram of hydrocarbons in the algal extracts. The chromatograms of C_{30} botryococcene and squalene standards were superimposed. (b) Changes in hydrocarbon contents per algal dry weight in static culture. (c) Changes in hydrocarbon contents per algal dry weight in aerated culture. (d) Changes in total hydrocarbon amount per 10 mL culture in static culture. (e) Changes in total hydrocarbon amount per 10 mL culture in aerated culture ($n = 3$, data is represented as mean $\pm$ S.E.M., *: $P < 0.05$, Student's t -test.). (f) Relative composition of a lower (C_{32}) to higher ($> C_{32}$) botryococcenes. Samples harvested from aerated culture at 2 weeks after inoculation were used. Similar results were obtained for static culture (not shown).

3.3. Effect of AHX on extracellular secondary carotenoid contents

As the color of AHX-treated cultures turned a slightly darker green than that of control cultures within a few days after inoculation, it was expected that more secondary carotenoids accumulated in the ECM of AHX-treated cultures. Indeed, secondary carotenoid contents significantly increased in AHX-treated cultures during the experimental period under static conditions. Carotenoid content was higher in AHX treated culture than control by 56% after 4 weeks of culture (Fig. 7a). Increase in secondary carotenoid contents was not observed with the addition of hypoxanthine (Sup. Fig 1b). Furthermore, the tendency to accumulate higher amounts of carotenoids induced by AHX was also observed in aerated cultures by 144% after 2 weeks of culture (Fig. 7b). Upon analyzing the extracts spectrophotometrically, absorption corresponding to chlorophyll *a* and *b* (645 ~ 663 nm) was not observed in the spectrum of extracts, indicating that this fraction did not include intracellular pigments such as chlorophylls or primary carotenoids.

To investigate the accumulation of secondary carotenoids in more detail, total secondary carotenoid fractions were subjected to column chromatography to separate individual classes of carotenoids and the resulting sub-fractions were subsequently analyzed by LC-AP/IMS to identify individual carotenoid species. The main component of the C₄₀ carotenoid fraction was identified as β -echinenone based on its retention time (14.8 min) on reversed phase HPLC, its molecular ion weight of m/z 551 as $[M+H]^+$, and its UV-VIS absorption spectrum ($\lambda_{max} = 460$) which were all identical to those of the authentic standard. The major component of the botryoxanthin fraction was eluted at 18.9 min and identified as botryoxanthin A, as it showed a molecular ion weight of m/z 1033 as $[M+H]^+$ and λ_{max} at 452 and 479 nm in the UV-VIS, consistent with data shown in previous work [11]. For the main component of the braunoxanthin fraction, braunoxanthin 1 was identified based on its retention time at 21.2 min, UV-VIS absorption spectrum ($\lambda_{max} = 465$) and mass fragment at m/z 1142 [12] identical to the authentic standard.

Thus, three major secondary carotenoid species, β -echinenone, botryoxanthin A, and braunixanthin 1 were detected in the ECM (Fig. 1a, b1 and c1). No primary carotenoids such as β -carotene or lutein were observed in the extracts, further suggesting that the carotenoid extracts were obtained from the ECM and not the intracellular region of the alga.

The relative amount of the carotenoid contents in the C_{40} carotenoid fraction including β -echinenone, the botryoxanthin fraction, and the braunixanthin fraction were individually determined, and all were observed to have significantly increased with AHX treatment (Fig. 7c,d). There are several examples showing that microalgae accumulate secondary carotenoids under nitrogen deficient conditions [33]. It is also known that secondary carotenoids accumulate in the B and L races of *B. braunii* during the stationary phase where algal growth is largely retarded due to nutrient exhaustion in the culture media, especially nitrogen sources [14, 15]. Extracellular secondary carotenoids of *B. braunii* may counteract stresses caused by exposure to excess visible or UV light. The hypothesis is indirectly supported by the fact that it accumulates during the stationary phase [10,18], at which point excess light can be harmful to photosynthetic machinery under nutrient deprivation. Although not specified to individual carotenoid species' properties, preceding work has also shown that *n*-hexane extracts of *B. braunii*, which corresponds to extracellular components, had antioxidant activity [34]. As non-invasive organic solvent extraction from *B. braunii* culture is reported [35,36], our findings might lead to a more productive way to harvest extracellular carotenoids as antioxidants in a sustainable manner without halting biomass growth. To verify this possibility, further characterization of the physical properties of structurally distinct TMS-conjugated carotenoids, botryoxanthins, and braunixanthins is warranted as they are the major components in the extracellular carotenoid fraction of *B. braunii* Showa strain. Given the fact that AHX induces secondary carotenoid accumulation in juice sac of citrus *Satsuma mandarin* [24], our

work further supports and expands the functionality of the relatively new bioactive natural compound, AHX, among diverse plant taxa.

It is interesting to note that β -echinenone may serve as the biosynthetic precursor of botryoxanthin A and braunixanthin 1 (Fig. 1a, b1 and c1). Stoichiometrically, one molecule of a TMS moiety derived from the triterpene biosynthetic pathway would be required for the synthesis of one molecule of botryoxanthin A or braunixanthin 1. This suggests that the biosynthesis of TMS, one of the triterpene hydrocarbons produced by the B race of *B. braunii*, seemed to be enhanced by AHX supplementation, while the production of other type of triterpenes, such as botryococcenes as the major components of free hydrocarbons, was retarded. Thus AHX can be used as a reagent to shift terpene biosynthesis in the B race of *B. braunii* from botryococcene production to methylsqualene derivative production without reducing growth rate significantly.

Fig. 7. Effect of AHX on extracellular secondary carotenoid contents of *B. braunii*. (a), (b): Total carotenoid contents under (a) static and (b) aerated culture condition; (c), (d): Contents of each fractionated carotenoid species. (c) C₄₀ carotenoid contents, (d) TMS-conjugating carotenoid contents. BTX: botryoxanthin fraction, BRX: braunioxanthin fraction. For determination of fractionated carotenoids, algal samples harvested at the second week of aerated culture were used. ($n = 3$, data is represented as mean $\pm$ S.E.M., *: $P < 0.05$, **: $P < 0.01$, Welch's t -test for (d), Student's t -test for (e).)

3.4. Residual nitrate content in culture medium

Decline in botryococcene accumulation and increased secondary carotenoid contents are generally observed during the stationary phase in a batch cultures of *B. braunii* [18]. A similar phenomenon was observed for AHX treatments in this study. As stationary phase can be induced by nutrient deprivation, especially nitrogen sources, physiological changes in the algae treated with AHX seemed to be caused by reduced uptake of nitrogen from culture media. To confirm this, residual nitrate contents in media from the cultures shown in Fig. 5b were measured. Residual nitrate content was higher in AHX-treated cultures (Fig. 8). In this context, evaluation of algal growth by increase of dry weight seems to be the most practical way for *B. braunii* at this moment because it is technically impossible to count individual cell numbers in colonies of *B. braunii*. On the other hand, increase of algal dry weight may not always reflect that of cell numbers if the weight of individual cells or the extracellular matrix become heavier. Therefore, the possibility that the higher residual nitrogen content observed here was due to lower cell division rates in the presence of AHX cannot be completely ruled out.

There were, however, no significant reductions in the dry weight of biomass in algae treated with AHX if compared with the control as shown in Fig. 5b. Thus, the higher residual nitrogen content likely implies that AHX might inhibit nitrate uptake, causing a nitrogen-depletion mimicking response in *B. braunii*. The continuous increase of algal biomass under such conditions with limited nitrogen-uptake may be attributed to the very high carbon/nitrogen ratio of this alga, such as 25-35 [37].

Fig. 8. Relative amount of residual nitrate in culture media 4 weeks after inoculation. Nitrate content of the media before inoculation of algae was set to 100%. Media for shaken culture were used ($n = 3$, data is represented as mean $\pm$ S.E.M.). Similar result was obtained in static culture (not shown).

In a previous study on the effects of AHX on rice seedlings, it was demonstrated that AHX enhanced the incorporation of ammonia/ammonium ions at roots and shoots [20]. When $^{15}\text{NH}_4\text{NO}_3$ or $\text{NH}_4^{15}\text{NO}_3$ was used as the sole nitrogen source for rice seedlings, increased accumulation of ^{15}N from $^{15}\text{NH}_4\text{NO}_3$ was observed in the presence of 1 mM of AHX especially at roots where expression of the gene coding for an aquaporin, *OsTIP2;1*, was up-regulated. This indicated that AHX could enhance uptake of ammonia/ammonium ions through the increased expression of aquaporins in rice. At present, information on the mechanism(s) for nitrogen assimilation in *B. braunii* has been very limited, although there was a report on the negative effects of ammonium chloride on the biosynthesis of botryococcenes [38]. Assuming that the acquisition of ammonia and nitrate from

culture media is carried out by some competitive mechanism in *B. braunii* and that AHX induces higher gene expression of some aquaporin-like protein(s) that enhance the uptake of ammonia in this alga (just like in the case of rice seedlings), it is plausible that nitrogen deficiency can be consequently induced since the media used for culturing *B. braunii* in this study did not contain any ammonium salts as nitrogen sources. The underlying mechanisms for the nitrogen deficiency-like phenomenon caused in *B. braunii* by AHX will be another interesting focus in future studies.

Conclusion

While AHX treatment significantly increased individual species of secondary carotenoids in the ECM of the *B. braunii* Showa strain, it also exhibited a tendency of decreasing extracellular hydrocarbon contents. The aforementioned change in terpene metabolism is often observed for *B. braunii* during its stationary phase, indicating that AHX induced a nitrogen-depletion mimicking response, which was consistent with reduced nitrate uptake observed in AHX-treated cultures. Despite this inhibitory effect, the biomass of AHX-treated *B. braunii* was only slightly reduced when compared to control cultures under aerated or mechanically shaken culture conditions. Furthermore, the correlation between reduced biomass and decreased total hydrocarbon content indicates that AHX did not have any significantly negative effects on production of any other algal metabolites other than botryococcenes that accumulated in the ECM. These seemingly contradicting results could be attributed to the high demand for nitrogen in order to maintain enzymatic systems needed for synthesizing large amounts of hydrocarbons for *B. braunii*, or intracellular nitrogen pools being sufficient enough to maintain growth during the experimental period used in this study. In static cultures, the biomass of *B. braunii* was significantly higher with AHX supplementation, possibly owing to the increased buoyancy of algal colonies, thus allowing more access to light and atmospheric CO₂.

To our knowledge, this is the first case in which terpene metabolism in *B. braunii* was modulated to result in higher carotenoid and lower hydrocarbon contents while maintaining its growth. Terpene-derived hydrocarbon biosynthesis of *B. braunii* is only known to be stimulated during its growth phase, but its detailed regulation still remains to be elucidated. As purchasable pure AHX is a still rather expensive reagent, it may not be economical to use AHX itself to increase carotenoid production in large-scale cultures for commercialization at this moment. On the other hand, 2-aza-8-oxohypoxanthine, an endogenous metabolite of AHX, has been known to show similar biological functions as AHX in rice [22]. Thus reducing the cost for the chemical synthesis of AHX or its derivatives will be necessary to make their applications for secondary carotenoid production in the alga more feasible. Our work has established a solid basis for further investigations on the underlying molecular mechanisms of terpene metabolism regulation in *B. braunii*, and several aspects of this are currently being undertaken.

Acknowledgments

This work was supported by CREST, JST (Japan Science and Technology Agency). We express our appreciation to Dr. VMEN Ferriols for critical discussions and comments on the manuscript.

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