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NOTE

Ethanol Enhances Astaxanthin Production by *Aurantiochytrium* sp. O5-1-1

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Abstract: Two-percent ethanol increased the astaxanthin productivity of heterotrophic microalgae *Aurantiochytrium* sp. O5-1-1 to 2.231 mg/L, 45-fold higher than under ethanol-free condition. Ethanol in the medium decreased at the same rate as spontaneous volatilization, suggesting that it was not a transient signaling factor but a continuous stress on the cells. The triply mutated strain OM3-3 produced 5.075 mg/L astaxanthin under 2% ethanol conditions. Furthermore, the astaxanthin accumulation of the mutant OM3-9 was 0.895 mg/g, which was 150-fold higher than that of strain O5-1-1 in ethanol-free condition. These results are beneficial for the commercial exploitation of carotenoids producing *Aurantiochytrium* spp.

Key words: astaxanthin, β-carotene, *Aurantiochytrium*, ethanol stress

1 Introduction

Most carotenoids are tetraterpenoids (C_{40}) composed of carbon-conjugated double bonds and hydrogen, and are broadly classified as carotenes without oxygen and xanthophylls with oxygen derived from hydroxyl and ketone groups[1]. The numbers of these conjugated double bonds, and hydroxyl and ketone groups determine the antioxidant potential of carotenoids. The singlet oxygen-scavenging capacity of astaxanthin (AST), a xanthophyll with particularly high antioxidant capacity, is about 40, 550, and 6,000 times greater than those of β-carotene, vitamin E, and vitamin C, respectively[2–4]. In fact, in *Haematococcus* spp., freshwater microalgae, accumulated AST diffuses to the cell periphery to protect them from photooxidative stress[5].

Carotenoid production processes have been reported in several plants, phytoplankton, and algae, and are commonly initiated by exposure to stresses such as nutrient deprivation, low temperature, high radiation, and high salinity[6–8]. Therefore, for large-scale AST production involving *Haematococcus* spp. and practical applications, a two-stage culture system consisting of biomass maximization and AST accumulation has been adopted. In general, high irradiance is required to induce AST production in the second stage. However, in practice, AST synthesis takes a long time and the risk of contamination is high, because the organism is cultivated outdoors using natural light that is unstable as to illumination[6]. Wen *et al.* reported that ethanol addition promoted AST production in *H. pluvialis* independent of light stimulation[9]. However, since *Haematococcus* spp. are autotrophs, light irradiation is essential during the growth period. Therefore, further improvements are needed to shorten the time and reduce the cost.

Thraustochytrids are unicellular microalgae that not only biosynthesize functional lipids such as docosahexaenoic acid (DHA), but also accumulate carotenoids such as β-carotene, AST, and canthaxanthin in their cells[10–12]. In addition, thraustochytrids are heterotrophic organisms, which means that they do not require light irradiation for culture, making mass cultivation easy. These characteristics indicate that thraustochytrids are promising new sources of AST instead of *Haematococcus* spp. In *Schizochytrium limacinum*, a thraustochytrid species, 0.8% butanol induced a decrease in fatty acid accumulation and increases in squalene, sterol, and carotenoid accumulation[13]. These effects are similar to the effects of ethanol in *H. pluvialis* shown by Wen *et al.*[9]. In this study, the effect of short-chain alcohols, from C_1 to C_4, on AST production by the AST high-producing *Aurantiochytrium* sp. O5-1-1 isolated from mangroves in Okinawa, Japan, was examined. In addition, AST production-enhancing mutant strains

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OM3-3 and OM3-9 were constructed using the O5-1-1 strain as a host.

2 Materials and Methods

2.1 Cultures

For isolation of Thraustochytrids, dYPD medium (0.2% glucose, 0.1% polypeptone, and 0.05% yeast extract in 50% artificial seawater) was used. GY medium (4% glucose and 1.5% yeast extract in 50% artificial seawater) was used to evaluate AST productivity. Predetermined concentrations of alcohols (methanol, ethanol, 1-propanol, and 1-butanol) were added to 4 mL GY liquid medium in which strain O5-1-1 was cultured for 24 hours, followed by shaking for 96 hours.

Liquid culture was performed at 25℃ with shaking (300 rpm). Solid media contained 1.5% agar.

2.2 Screening of astaxanthin high-producing thraustochytrids

A small piece of mangrove leaf from Okinawa, Japan, and sterilized pine pollen were inoculated into dYPD liquid medium containing 100 μg/mL chloramphenicol, followed by incubation at room temperature for 2 days. The floating pine pollen was streaked onto dYPD agar medium and incubated for 2 days. The redder strains were visually sorted from the growing colonies.

Isolated strain O5-1-1 was identified by sequencing of the 18S ribosomal DNA region amplified with a pair of primers, EukA: 5'-AACCTGGTTGATCCTGCCAGT-3' and EukB: 5'-TGATCCTTCTGCAGGTTCACCTAC-3'[14]. Furthermore, EukA, EukB, Euk-550R (5'-GAATTACCGCGGCTGCTGGC-3'), Euk-950R (5'-ATCCCCTAACTTTCGTTCTTG-3'), and Euk-1500R (5'-CCAGAACGTCTAAGGGCATCACAG-3') were used as primers for sequencing. The genome of strain O5-1-1 used for PCR was extracted by the alkaline-thermal shock procedure. Amplified PCR products were sequenced (Macrogen Inc., Japan) and analyzed using the BLASTn algorithm of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/).

2.3 Carotenoid analysis

Cell pellets from 4 mL aliquots of medium collected by centrifugation were transferred to 2 mL tubes and freeze-dried overnight. The dried cells were resuspended in 500 μL of methyl *tert*-butyl ether (MTBE) and 500 μL of methanol, and then crushed with glass beads. The supernatant after crushing was collected and used for high-performance liquid chromatography (HPLC) analysis. A reversed-phase column, YMC Carotenoid 4.6 ID × 150 mm (YMC Co., Ltd., Japan), was used for carotenoid analysis. Methanol/MTBE/H_2O (81/15/4) was used as mobile phase A, and Methanol/MTBE/H_2O (8/90/2) as mobile phase B. The flow rate was set to a total of 1.0 mL/min, and the gradient conditions were such that the percentage of mobile phase B increased from 0% to 89% within 40 minutes of the start of analysis. The detection absorbance wavelength, column temperature, and sample injection volume were fixed at 470 nm, 30℃, and 20 μL, respectively. Calibration curves were prepared using AST (FUJIFILM, Japan) and β-carotene (FUJIFILM) standards to quantify these carotenoids from the cells. All experiments were performed three times. Data were analyzed by one-way repeated measures ANOVA, and significant differences between the groups were determined by Bonferroni method ($p < 0.01$) using R version 4.2.1.

2.4 Fatty acid analysis

The preparation of fatty acid methyl esters (FAMEs) and their analysis by gas chromatography (GC) was described previously[15]. Total lipids of dried cells were transmethylated with 10% methanolic HCl for 4 hours at 55℃. The resulting FAMEs were extracted using *n*-hexane, concentrated, and analysed by GC-2025 gas chromatography (Shimadzu, Japan) on a TC-70 capillary column (GL Science, Japan). Fatty acid quantification was performed using linoleic acid (18:2ω6; Tokyo Chemical Industry, Japan) as an internal standard.

2.5 Residual ethanol measurement

Residual ethanol in the culture supernatant was determined by GC analysis using a DB-SELECT 624UI capillary column (Agilent Technologies, Japan). The temperature of the column was initially set at 40℃ for 20 minutes, and then increased to 200℃ at 10℃/min and kept there for 20 minutes. The temperature of the vaporization chamber and the detector were both 260℃. Helium was used as the carrier gas. Calibration curves were prepared using ethanol (>99.9%) (Kishida Chemical, Japan) standard.

2.6 Mutagenesis

A final concentration of 500 μg/mL *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine was added to 1×10^7 cells suspended in 50 mM Tris-maleate buffer (pH 7.5), followed by incubation at 25℃ for 60 min. Three volumes of 10% (w/v) sodium thiosulfate was then added to stop the reaction. The viable cells washed twice with sterile water were streaked onto GY agar medium and incubated under fluorescent light for 2 days, after which strongly red colonies were visually selected. Mutational treatment effects were assessed by less than 10% survival and diversification of colony size.

3 Results and Discussion

3.1 Effect of alcohols on carotenoid production

The C_1-C_4 alcohols given to the cells cultured for 24 hours all enhanced the production of AST (Table 1). In particular, 2% ethanol caused AST productivity and AST accumulation to reach 2.23 mg/L (45-fold higher than control) and 0.242 mg/g (73-fold higher than control), respectively (Table 1). The carbon number of alcohols was proportional to the induction of AST production as well as growth inhibition.

AST accumulation under 0.5% butanol conditions reached 0.065 mg/g, which was generally consistent with the results of Zhang *et al.*[13]. On the other hand, 2% ethanol induced 4-fold higher AST accumulation than 0.5% butanol. These results suggested that ethanol could more effectively induce AST production in strain O5-1-1, as in *H. pluvialis*.

3.2 Time course of carotenoid production under ethanol-addition and -free conditions

The production of AST and β-carotene in strain O5-1-1 under ethanol-free conditions started after the stationary phase, reaching 0.086 mg/L and 0.116 mg/L, respectively, at 144 hours culture (Table 2). While 2% ethanol significantly increased the production of carotenoids immediately after addition. AST and β-carotene production reached 2.063 mg/L and 0.368 mg/L at 120- and 72-hours culture, respectively (Table 2). These results suggest that ethanol comprehensively up-regulates the carotenoid production process, including the β-carotene synthesis pathway and the conversion pathway to AST.

The increase in carotenoid production on ethanol addition decreased the accumulated fatty acids (Table 2). This result is consistent with the effect of butanol reported by Zhang *et al.*[13]. Residual ethanol after the stationary phase decreased at the same rate as spontaneous volatilization, suggesting that strain O5-1-1 does not utilize ethanol as a carbon source, carotenoid material, or signaling factor, and continued to be under ethanol stress (Fig. 1). These results suggest that strain O5-1-1 that have reached stationary phase prioritizes defense against alcohol by AST production rather than metabolism.

3.3 Astaxanthin productivity-enhancing mutants

Mutant strain OM1-4 with enhanced AST productivity was constructed using strain O5-1-1 as a host. In addition, strain OM1-4 was mutated to obtain the OM2-4 and OM2-5 strains. The OM3-3 and OM3-9 strains were parented to OM2-5 and OM2-4 strains, respectively. Under the 2% ethanol conditions, the AST productivity of triply mutated strain OM3-3 reached 5.075 mg/L, which was 2.3-fold higher than that of strain O5-1-1 (Table 3). Furthermore, the AST accumulation by strain OM3-9 was 0.895 mg/g, which was 150-fold higher than that by strain O5-1-1 under ethanol-free conditions (Tables 2 and 3).

The AST productivity of all selected mutants was higher

Table 1 Effects of alcohol on carotenoids production in *Aurantiochytrium* sp. O5-1-1.

Condition*		Astaxanthin (mg/L)	β-carotene (mg/L)	Dry cell weight (g/L)
Control		0.052 ± 0.003^{a}	n.d.	15.00 ± 0.30
Methanol	0.5%	$0.106 \pm 0.006^{a,b}$	$0.087 \pm 0.011^{a,b}$	15.25 ± 0.69
	1.0%	$0.148 \pm 0.020^{a,b}$	$0.078 \pm 0.011^{a,b}$	14.87 ± 0.50
	2.0%	$0.327 \pm 0.029^{b,d}$	$0.099 \pm 0.002^{a,b,c}$	14.23 ± 0.47
Ethanol	0.5%	$0.152 \pm 0.010^{a,b}$	$0.156 \pm 0.023^{d,e}$	15.75 ± 0.40
	1.0%	0.296 ± 0.024^{b}	0.178 ± 0.020^{d}	15.45 ± 0.58
	2.0%	2.231 ± 0.050^{c}	$0.123 \pm 0.005^{a,e}$	9.22 ± 0.43
1-Propanol	0.5%	0.550 ± 0.055^{d}	0.056 ± 0.003^{b}	9.25 ± 0.59
	1.0%	1.592 ± 0.168^{e}	n.d.	6.62 ± 0.32
	2.0%	0.035 ± 0.031^{a}	n.d.	6.87 ± 0.42
1-Butanol	0.5%	0.952 ± 0.072^{f}	$0.135 \pm 0.008^{c,d,e}$	14.70 ± 0.00
	1.0%	n.d.**	n.d.	7.87 ± 0.05
	2.0%	n.d.	n.d.	11.27 ± 1.58

* Alcohols were added to the GY medium in which the strain O5-1-1 was cultured for 24 hours, followed by shaking for 96 hours. All data are the means ± standard deviation (SD) for triplicate samples.

** "n.d.", not detected.

Mean values not sharing a common letter are significantly different between conditions for same carotenoid ($p < 0.01$), estimated by Bonferroni method following one-way ANOVA.

Table 2 Time course of induction of carotenoids production in *Aurantiochytrium* sp. O5-1-1 by 2% ethanol.

Total incubation time (h)*		Astaxanthin (mg/L)	β-carotene (mg/L)	Fatty acid production (g/L)	Dry cell weight (g/L)
Ethanol-free	24	n.d.**	0.012 ± 0.001^{a}	5.51 ± 1.88	10.43 ± 0.09
	48	n.d.	0.073 ± 0.000^{a}	5.95 ± 0.31	12.46 ± 0.74
	72	0.022 ± 0.006^{a}	0.099 ± 0.007^{a}	5.52 ± 0.24	12.39 ± 0.22
	96	0.038 ± 0.001^{a}	0.100 ± 0.009^{a}	4.89 ± 0.09	12.14 ± 0.11
	120	0.053 ± 0.005^{a}	0.116 ± 0.003^{a}	4.86 ± 0.16	11.74 ± 0.22
	144	0.086 ± 0.026^{a}	0.116 ± 0.012^{a}	3.77 ± 0.54	10.54 ± 0.28
	168	0.073 ± 0.001^{a}	0.111 ± 0.007^{a}	3.77 ± 0.11	10.64 ± 0.48
2% ethanol	48	0.134 ± 0.023^{a}	0.316 ± 0.039^{b}	5.96 ± 0.38	12.23 ± 0.40
	72	0.791 ± 0.091^{b}	0.368 ± 0.006^{c}	3.47 ± 0.26	10.20 ± 0.13
	96	1.864 ± 0.027^{c}	$0.214 \pm 0.005^{d,e}$	1.93 ± 0.29	7.38 ± 0.09
	120	2.063 ± 0.117^{c}	0.261 ± 0.006^{d}	0.63 ± 0.11	6.29 ± 0.27
	144	1.781 ± 0.261^{c}	$0.234 \pm 0.016^{d,e}$	0.45 ± 0.17	5.33 ± 0.33
	168	1.223 ± 0.014^{d}	0.197 ± 0.003^{e}	0.33 ± 0.07	3.87 ± 0.20

* Ethanol was added to the GY medium in which the strain O5-1-1 was cultured for 24 hours, followed by shaking. All data are the means ± standard deviation (SD) for triplicate samples.

** "n.d.", not detected.

Mean values not sharing a common letter are significantly different between conditions for same carotenoid ($p < 0.01$), estimated by Bonferroni method following one-way ANOVA.

Table 3 Carotenoids productivity of *Aurantiochytrium* sp. O5-1-1 mutants.

Condition	Strain	Parent	Astaxanthin (mg/L)	β-carotene (mg/L)	Dry cell weight (g/L)
Ethanol-free	O5-1-1		0.066 ± 0.005^{a}	$0.122 \pm 0.002^{a,b}$	10.98 ± 0.18
	OM1-4	O5-1-1	$0.529 \pm 0.019^{a,b}$	$0.249 \pm 0.012^{c,d}$	10.21 ± 0.37
	OM2-4	OM1-4	$0.726 \pm 0.061^{a,b}$	0.412 ± 0.046^{e}	10.11 ± 0.20
	OM3-9	OM2-4	$1.663 \pm 0.100^{c,d}$	0.086 ± 0.002^{a}	9.27 ± 0.06
	OM2-5	OM1-4	0.882 ± 0.070^{b}	0.317 ± 0.014^{f}	9.47 ± 0.10
	OM3-3	OM2-5	$1.114 \pm 0.098^{b,c}$	$0.157 \pm 0.014^{b,g}$	9.75 ± 0.05
2% ethanol	O5-1-1		2.148 ± 0.178^{d}	$0.312 \pm 0.014^{c,f}$	6.68 ± 0.16
	OM1-4	O5-1-1	3.306 ± 0.205^{e}	0.537 ± 0.012^{h}	7.41 ± 0.15
	OM2-4	OM1-4	3.972 ± 0.451^{e}	$0.122 \pm 0.017^{a,b}$	6.62 ± 0.37
	OM3-9	OM2-4	5.050 ± 0.170^{f}	$0.147 \pm 0.014^{a,b,i}$	5.64 ± 0.12
	OM2-5	OM1-4	3.801 ± 0.279^{e}	$0.183 \pm 0.013^{b,i}$	6.25 ± 0.18
	OM3-3	OM2-5	5.075 ± 0.329^{f}	$0.203 \pm 0.014^{d,g,i}$	6.18 ± 0.26

Two percent ethanol was added to the GY medium in which the strain O5-1-1 and mutants were cultured for 24 hours, followed by shaking for 96 hours. All data are the means ± standard deviation (SD) for triplicate samples.

Mean values not sharing a common letter are significantly different between conditions for same carotenoid ($p < 0.01$), estimated by Bonferroni method following one-way ANOVA.

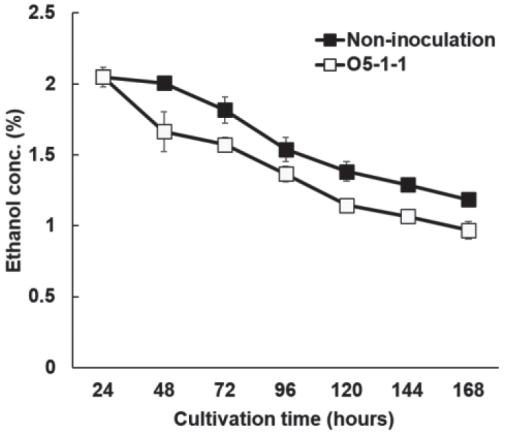

Fig. 1 Time course of ethanol level in the medium with (white squares) or without (black squares) *Aurantiochytrium* sp. O5-1-1 inoculation.

than that of O5-1-1 in the absence of ethanol (Table 3), and was synergistically promoted by 2% ethanol (Table 3). Thus, enhancement of carotenoid production by ethanol was also effective in the mutant strains.

4 Conclusion

2% ethanol was more effective than other short-chain alcohols, and increased the AST production by *Aurantiochytrium* sp. O5-1-1 to 2.063 mg/L at 96 hours after addition. Furthermore, the increased production of β-carotene, a precursor of AST, suggested that ethanol promoted the entire carotenoid synthesis mechanism. With triply mutated strains, OM3-3 and OM3-9, AST productivity reached 5.075 mg/L and AST accumulation 0.895 mg/g, respectively. The AST productivity of all selected mutants was higher than that of O5-1-1 under ethanol-free conditions and synergistically enhanced by 2% ethanol. These results will be beneficial for the commercial exploitation of carotenoid-producing *Aurantiochytrium* spp.

Conflict of Interest

The authors declare no competing conflict of interest.

Author Contributions

TS and ES designed the research. YI and NM carried out the experimental work, and analysed and interpreted the data. TS and ES recommended and edited the paper. All authors contributed to the article and approved the submitted version.

References

1) Ellison, S.L. Carotenoids; Physiology. in *Encyclopedia of Food and Health* (Caballero, B.; Finglas, P.M.; Toldra, F. eds.), Elsevier, Amsterdam, pp. 670-675 (2016). doi: 10.1016/B978-0-12-384947-2.00120-3
2) Miki, W. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **63**, 141-146 (1991). doi: 10.1351/pac199163010141
3) Shimizu, N.; Goto, M.; Miki, W. Carotenoids as singlet oxygen quenchers in marine organisms. *Fisheries Sci.* **62**, 134-137 (1996).
4) Nishida, Y.; Yamashita, E.; Miki, W. Quenching activities of common hydrophilic and lipophilic antioxidants against singlet oxygen using chemiluminescence detection system. *Carotenoid Sci.* **11**, 16-20 (2007). doi: 10.11501/10996240
5) Ota, S.; Morita, A.; Ohnuki, S.; Hirata, A.; Sekida, S. *et al.* Carotenoid dynamics and lipid droplet containing astaxanthin in response to light in the green alga *Haematococcus pluvialis*. *Sci. Rep.* **8**, 5617 (2018). doi: 10.1038/s41598-018-23854-w
6) Lorenz, R.T.; Cysewski, G.R. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **18**, 160-167 (2000). doi: 10.1016/S0167-7799(00)01433-5
7) Quian-Ulloa, R.; Stange, C. Carotenoid biosynthesis and plastid development in plants: The role of light. *Int. J. Mol. Sci.* **22**, 1184 (2021). doi: 10.3390/ijms22031184
8) Ram, S.; Mitra, M.; Shah, F.; Tirkey, S.R.; Mishra, S. Bacteria as an alternate biofactory for carotenoid production: a review of its applications, opportunities and challenges. *J. Funct. Foods* **67**, 103867 (2020). doi: 10.1016/j.jff.2020.103867
9) Wen, Z.; Liu, Z.; Hou, Y.; Liu, C.; Gao, F. *et al.* Ethanol induced astaxanthin accumulation and transcriptional expression of carotenogenic genes in *Haematococcus pluvialis*. *Enzyme Microb. Technol.* **78**, 10-17 (2015). doi: 10.1016/j.enzmictec.2015.06.010
10) Aasen, I.M.; Ertesvåg, H.; Heggeset, T.M.B.; Liu, B.; Brautaset, T. *et al.* Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids. *Appl. Microbiol. Biotechnol.* **100**, 4309-4321 (2016). doi: 10.1007/s00253-016-7498-4
11) Gupta, A.; Singh, D.; Barrow, C.J.; Puri, M. Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. *Biochem. Eng. J.* **78**, 11-17 (2013). doi: 10.1016/j.bej.2013.04.028
12) Park, H.; Kwak, M.; Seo, J.; Ju, J.; Heo, S.; Park, S. Enhanced production of carotenoids using a thraustochytrid microalgal strain containing high levels of docosahexaenoic acid-rich oil. *Bioprocess Biosyst. Eng.* **41**, 1355-1370 (2018). doi: 10.1007/s00449-018-1963-7

13) Zhang, K.; Chen, L.; Liu, J.; Gao, F.; He, R. *et al.* Effects of butanol on high value product production in *Schizochytrium limacinum* B4D1. *Enzyme Microb. Technol.* **102**, 9-15(2017). doi: 10.1016/j.enzmictec.2017.03.007
14) von der Heyden, S.; Chao, E.E.; Vickerman, K.; Cavalier-Smith, T. Ribosomal RNA phylogeny of bodonid and diplonemid flagellates and the evolution of Euglenozoa. *J. Eukaryot. Microbiol.* **51**, 402-416(2004). doi: 10.1111/j.1550-7408.2004.tb00387.x
15) Sakamoto, T.; Sakuradani, E.; Okuda, T.; Kikukawa, H.; Ando, A. *et al.* Metabolic engineering of oleaginous fungus *Mortierella alpina* for high production of oleic and linoleic acids. *Bioresour. Technol.* **245**, 1610-1615(2017). doi: 10.1016/j.biortech.2017.06.089

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