BIOTECHNOLOGY LETTERS
Volume 16 No.2 (February 1994) pp.133-138
Received as revised 12th January

HYPER-ACCUMULATION OF ASTAXANTHIN IN A GREEN ALGA *HAEMATOCOCCUS PLUVIALIS* AT ELEVATED TEMPERATURES

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SUMMARY

When a green alga *Haematococcus pluvialis* was cultivated at 30°C, astaxanthin production was 3-fold more increased than at 20°C. With acetate supplementation to 30°C culture, the alga synthesized over 2-fold more carotenoid than without addition. Tiron, a radical scavenger, however, severely blocked the stimulated carotenogenesis, suggesting that endogenously generated active oxygen was responsible for the highly stimulated carotenogenesis. From these results, possible roles of the elevated cultivation temperatures for hyper-accumulation of astaxanthin were discussed.

INTRODUCTION

A unicellular green alga *Haematococcus pluvialis* can synthesize astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione), which has been utilized in fish aquaculture as pigmentation source. Since this ketocarotenoid has been shown to possess higher antioxidative activity than β-carotene and α-tocopherol (Miki, 1991), it could be applied as a food colorant, or in medical use. We have been studying regulation of the carotenoid biosynthesis in *Haematococcus* to carry out high astaxanthin production by the alga. Astaxanthin is massively synthesized only in a resting stage termed as a cyst, which was induced under unfavorable growth conditions such as nitrogen deficiency (Boussiba and Vonshak, 1991, Kakizono *et al.*, 1992). We have recently demonstrated that five kinds of active oxygen species (singlet oxygen, superoxide anion radical, H_2O_2, peroxy radical, and hydroxyl radical) were all capable of stimulating carotenogenesis in acetate-induced cysts (Kobayashi *et al.*, 1993a). For cyst stage induction, it can be postulated that an elevated culture temperature should provide non-growth condition. Moreover, since active oxygens can be

endogenously generated from photosynthesis (Halliwell and Gutteridge, 1989), it seems plausible to assume that relatively high culture temperature may also lead enhanced formation and/or higher reactivities of active oxygens in the algal cell. In the present study, we have examined carotenoid formation under elevated temperatures over 20°C, and shown that endogenously produced active oxygens were essential for hyper-accumulation of astaxanthin in *Haematococcus* at elevated temperatures

MATERIALS AND METHODS

Algal Cultivation A unicellular green alga *H. pluvialis* NIES-144 obtained from the National Institute for Environmental Studies, Tsukuba, Japan was cultivated in basal medium as acetate for carbon source at 15 mM (Kobayashi *et al.*, 1991). To examine effect of culture temperature, a 200 ml of Erlenmeyer flask containing 100 ml of basal medium was incubated at the indicated temperature under 2.0 klux illumination (ca. 26 $\mu E \cdot m^{-2} \cdot s^{-1}$) with reciprocal shaking in an illuminated thermostat chamber. Preculture was incubated at 20°C as described. For supplementation culture (Kobayashi *et al.*, 1993a), sodium acetate (2.25 M, pH 7.0) and/or ferrous sulfate (22.5 mM, pH 1.5) were added to 45 mM and 0.45 mM, respectively at 4-d cultivation, and the culture was further incubated for 6 d. A radical scavenger, Tiron (sodium 1,2-dihydroxybenzene 3,5-disulfonate), and a singlet oxygen quencher, DABCO (1,4-diazabicyclo[2.2.2]octane) were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Analyses Chlorophylls were determined as described previously (Kobayashi *et al.*, 1991). Total carotenoids extracted with 90 % acetone were expressed as astaxanthin because total forms of astaxanthin (monoester, diester, and free forms) were the major carotenoids over 85–95 % in the algal extract, which was confirmed on TLC analysis (Kobayashi *et al.*, 1991). Cell concentration was measured either as cell number by haemacytometer, or as dry cell weight after filtration on 0.45-µm nitrocellulose membrane. All the analyses were carried out for duplicate cultures, and their averages were employed.

RESULTS AND DISCUSSION

Effect of Cultivation Temperatures Under mixotrophic growth condition (Kobayashi *et al.*, 1992) as acetate for carbon source, the alga was cultivated at various temperatures from 20 to 35°C to examine effect of temperature on cell growth and carotenoid formation. The alga carried out vegetative cell growth at 20°C throughout the cultivation period for 8 d (Fig.1). The result indicated that no morphological change from green vegetative cells to enlarged red cyst cells took place, resulting in poor carotenoid formation (Fig.1b). In good contrast, at elevated temperatures over 30°C, vegetative growth was drastically retarded, while astaxanthin formation at 30°C and 33°C (19.0, and 22.6 mg/*l* culture, respectively) was 2.5 to 3 times higher than

cultivated at 20°C (8.0 mg/*l*). Due to lower cell number at these higher temperatures, carotenoid production was even more improved from 15 to 20 times as a content basis (Fig.1b). At these elevated temperatures, it was observed that the algal cells have rapidly turned into large dark red cysts within 2 d. Chlorophyll formation was reduced when cultivated at over 25°C (Fig.1c). At 35°C, the alga was lysed soon after inoculation.

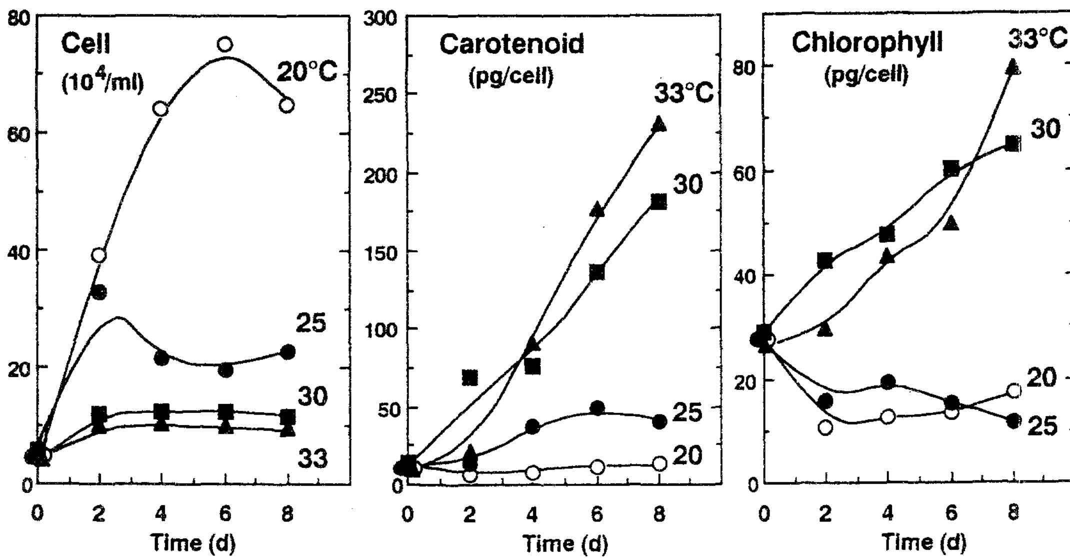

Figure. 1 Effect of Elevated Temperatures on Cell Growth (a), Carotenoid (b), and Chlorophyll (c) Formation in *Haematococcus*.

It has been shown that *Haematococcus* tends to accumulate a large amount of astaxanthin via cyst formation under unfavorable growth conditions: deficiencies of nitrogen (Boussiba and Vonshak, 1991, Kakizono *et al.*, 1992), of sulfate, and of phosphate, and the presence of cell growth inhibitor (Boussiba and Vonshak, 1991). Thus, it was likely that growth interfered by such adverse environments would result in induction of cyst formation as suggested in high carbon/nitrogen medium (Kakizono *et al.*, 1992), and then massive astaxanthin formation would be triggered in the induced cyst cells (Kobayashi *et al.*, 1993a). Essential roles of oxidative stress in carotenoid biosynthesis have recently been proposed by Kobayashi *et al.* (1993a), carotenogenesis in *Haematococcus* was activated by oxidative stress without *de novo* protein synthesis. Shaish *et al.* (1993) has also shown that induction of β-carotene accumulation in a green alga *Dunaliella*

bardawil by high light was replaceable with addition of active oxygen species under low light. Therefore, it can be indicated that elevated culture temperatures may play two critical roles in carotenogenesis of *Haematococcus.* First, normal vegetative growth should be interrupted at a growth impermissible temperature, and second higher temperature would stimulate formation and/or reactivity of active oxygen species that are endogenously generated from photosynthesis (Halliwell and Gutteridge, 1989), which eventually contributed to hyper-accumulation of astaxanthin.

Supplementation of Radical Scavengers and Active Oxygens It was of interest whether exogenously supplied oxidative stress would lead higher carotenoid accumulation even at 30°C. Since Fe^{2+} is known to generate hydroxyl radical (HO•) from Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow OH^- + HO\bullet + Fe^{3+}$ (Halliwell and Gutteridge, 1989), acetate, Fe^{2+}, and/or H_2O_2 were added to 4-d culture incubated at 30°C. As shown in Fig.2, it was found that with acetate addition alone, carotenoid formation at 30°C was over two-fold increased than no addition, and Fe^{2+} and/or H_2O_2 plus acetate were only slightly stimulative in carotenogenesis at 30°C. The result implied that intracellular active oxygen level was not a limiting factor for carotenoid formation at 30°C unlike at 20°C. Moreover, if this is the case, radical scavengers or quenchers would inhibit hyper-accumulation of carotenoid as shown previously at 20°C (Kobayashi *et al.*, 1993a). With addition of Tiron, a scavenger for superoxide anion radical (O_2^-), carotenoid formation at 30°C was drastically reduced (Fig.2).

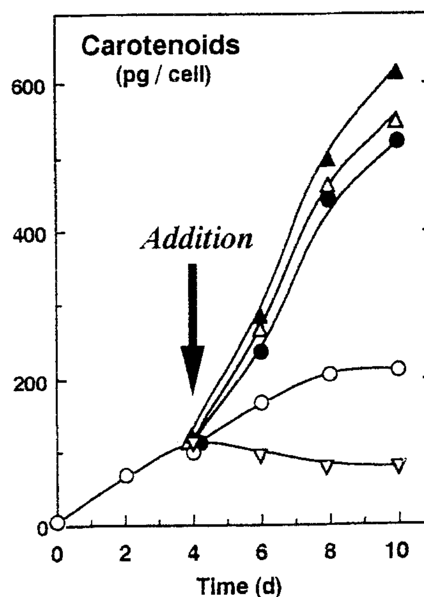

Figure. 2 Effect of Supplementation of Acetate, Fe^{2+}, H_2O_2, and/or Tiron on Carotenoid Formation of *Haematococcuss* to 30 °C Culture. Addition at 4 d: ●, acetate (45 mM); Δ, acetate+Fe^{2+} (0.45mM); ▲, acetate +Fe^{2+}+H_2O_2 (0.1μM); ∇, acetate+Tiron (1 mM); O, no addition. Residual acetate at 4 d before the addition were 3.0±0.7mM. Cell numbers were between 6.2 to 7.8 (x10^4 cell / ml) at 8 d culture.

Table 1. Effect of Scavengers and Quencher on Carotenoid Formation Cultivated at 30°C for 8 d Culture.

Addition* (mM)		Cell (10^4/ml)	Carotenoid (pg/cell)
No addition		7.0	230.8
Tiron	0.1	7.6	216.8
	1.0	6.6	**133.3**
	10.0	4.5	**128.0**
DABCO	0.1	7.3	203.8
	1.0	6.7	210.1
	10.0	4.4	59.1
Iodide	1.0	7.4	227.0

* Together with the inoculum.

Table 2. Independence of Light Intensity on Carotenoid Formation with Acetate and Fe^{2+} Addition at 30°C Culture.

Light intensity	Addition	Carotenoid (pg/cell)	Chlorophyll (pg/cell)
Low*	None	181.0	48.1
	Acetate+Fe^{2+}	**563.9**	87.1
High#	None	235.3	62.2
	Acetate	574.8	78.5
	Acetate+Fe^{2+}	**554.8**	75.7

* Low: 2.0 klux (ca. 26 $\mu E \cdot m^{-2} \cdot s^{-1}$).

High: 9.0 klux (ca. 103 $\mu E \cdot m^{-2} \cdot s^{-1}$).

Tiron itself has been verified uninhibitory to carotenoid biosynthesis because it could specifically block carotenogenesis activated by O_2^-, but neither by H_2O_2, nor by singlet oxygen ($^1O_2$) (Kobayashi *et al.*, 1993a). Among other scavengers and quenchers, however, DABCO as an $^1O_2$ quencher, and iodide as a HO• scavenger were not effective in blocking carotenoid formation at 1 mM level (Table 1). Thus, it seems very likely that active oxygen species, especially O_2^- that can be endogenously generated in photosynthesis would be involved in stimulated carotenogenesis at the elevated temperatures.

Effect of Light Intensity It has been shown that activation of carotenogenesis in *Haematococcus* has required optimal levels of light intensity between 6.0 klux (ca. 68 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 11.0 klux (ca. 125 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Kobayashi *et al.*, 1993 a, 1993b). However, as shown in Table 2, there were only slight differences for carotenoid formation with the supplementations at 30 °C between high (9.0 klux) and low light intensities (2.0 klux), which was the standard condition in this study. From this result, it seemed that light energy was not so critical for generation of active oxygen species, and/or their reactivity at this elevated temperature than at 20°C, although in the dark negligible carotenoid was formed at 20 and 30°C.

From the results obtained in this study, we were able to attain high levels of astaxanthin production by cultivating at 30°C with supplementation of acetate, Fe^{2+} and H_2O_2. The hyper-accumulation of the carotenoid (613 pg/cell) has reached nearly ten times higher than our previous values (Kobayashi *et al.*, 1993a) as a cellular content basis. It is thus anticipated to carry out the algal culture in a larger scale for the carotenoid production. For this purpose, less light requirement at 30°C culture would provide advantageous operation for photobioreactor to reduce expense of illumination.

ACKNOWLEDGMENT

This work was supported by the Shorai Foundation for Science and Technology (1993), and Grants-in-Aid for scientific research (05304010) from the Ministry of Education, Science and Culture of Japan.

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