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Effects of temperature on the astaxanthin productivity and light harvesting characteristics of the green alga *Haematococcus pluvialis*

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The green alga *Haematococcus pluvialis*, which accumulates astaxanthin at an optimal temperature of 20°C, was cultivated under temperatures of 20°C, 23.5°C, 27°C, and 30.5°C, in order to assess the effects on algal metabolism during the growth phase. The culture growth rate declined with above-optimal increases in temperature, and the final maximum cell concentration at 30.5°C reached only 35% of that attained at 20°C. On the contrary, the biomass productivity was increased under all the high-temperature conditions, probably reflecting the metabolism switch from cell duplication to energy accumulation that is typically observed in algal cultures subjected to environmental stress. Moreover, an increase in the light-harvesting capability of the alga was observed by means of the total pigment balance and the photosynthesis-intensity (PI) curve measured under the different cultivation conditions. Cultures kept at higher temperatures were able to better harvest and utilize the impinging light due to photo-acclimation. Finally, the differences in the astaxanthin metabolism were elucidated by subjecting the cultures to nitrogen starvation at 20°C and 27°C. In the culture at 27°C, a 1.4-fold increase in the astaxanthin productivity was observed when compared to that at 20°C, and the latter required almost two-fold more energy for the astaxanthin production compared with the 27°C culture.

[**Key words:** *Haematococcus pluvialis*; Photobioreactor; Astaxanthin; Photosynthetic efficiency]

Haematococcus pluvialis is a green alga widely accepted as one of the best sources of the keto-carotenoid astaxanthin. Astaxthanthin, which can also be found in marine organisms such as krill, shrimp, and crab (1), is widely used as an additive in foods, cosmetics, and nutraceuticals due to its high antioxidant activity (2). Astaxanthin production in *H. pluvialis* starts with the morphological change from the motile green vegetative cells towards red cysts lacking flagella. This morphological change can be induced by several stresses such as high temperature, high light intensity (3), and nutrient deficiency (4). The astaxanthin accumulation is believed to be a response of the cells to protect themselves from the oxidative stress caused by the reactive oxygen species (ROS) from the excessive reducing power that is accumulated in the photosynthetic electron transport chain (5,6).

The industrial application of algae to produce astaxanthin has many problems, the most relevant being the large amount of energy it requires, as this directly affects the final price of the product. The algal biosynthesis of astaxanthin is almost four times more expensive than the chemical synthesis (7). One of the main sources of energy required in photobioreactors (PBRs) is the electricity that is necessary to keep the culture at its optimal growth temperature. In order to reduce the production cost, we focused on the investigation of a less energy-intensive cultivation temperature. The cultivation temperature significantly affects algae. Under suboptimal temperature conditions, photosynthetic activity is severely reduced when compared to the optimal conditions (8). Latala (9) reports that in the case of algae grown under very high temperature conditions, the growth is at least partially impaired or even strongly hindered. This means that both low and high temperatures exert an inhibitory effect on algae, which necessitates a maintenance of cultivation temperature at optimal conditions. In particular, *Haematococcus* cultures are shown to be quite sensitive to temperature changes (10–12). The optimal temperature for the *H. pluvialis* strain used in the present study was 20°C; maintaining such a low temperature under high illumination conditions requires increased energy, and, therefore, higher cultivation temperatures would be desirable if the object is to decrease production costs. In fact, many industrial outdoor cultivation operations are conducted in tropical climatic regions where, on average, light intensity and temperature are too high for uncontrolled algal cultivation. In an effort to increase efficiency of the overall astaxanthin production, in the present study we first attempted to elucidate how temperature affects cell growth. We then demonstrated positive effects of appropriate temperature selection by means of an energetic assessment toward outdoor cultivations using PBRs.

MATERIALS AND METHODS

Strain and culture conditions The strain used in the present study was *H. pluvialis* NIES-144 obtained from the Microbial Culture Collection of the National Institute for Environmental Studies (NIES; Japan), which was isolated in Hokkaido, Japan. This strain is believed to have an optimum cultivation temperature of approximately 20°C.

The seeding culture was prepared by using an exponentially growing culture of *H. pluvialis*, which was concentrated by centrifugation and resuspended in C

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medium (13) up to a final concentration of 3.3×10^5 cells ml^{-1} to be subdivided in 10-ml aliquots for storage. The tubes were maintained photoautotrophically at 20°C in an incubator as suggested by the culture collection institute. Light was supplied by means of an 18 W cool white fluorescent tube (FL20SS·ECW/18X; Panasonic, Japan) and the intensity was regulated to 10 μmol m^{-2} s^{-1} by using white semi-transparent sheets. Light intensity was measured with a quantum photometer (LI-250A; LI-COR, USA) equipped with a cosine-corrected flat probe (LI-190; LI-COR). No culture older than 1 month was used in the present study to ensure that only the best vital cells would be used for the growth experiments. Prior to the inoculation in the PBR, the seeding culture was grown for 4 days in unshaken Erlenmeyer flasks using Kobayashi basal medium (14). The culture concentration at the end of this step reached $6.5 \pm 0.3 \times 10^5$ cells ml^{-1}. The temperature was kept constant by means of a thermostatic bath and the light was again supplied by means of an 18 W cool white fluorescent tube. Light intensity was regulated to 70 μmol m^{-2} s^{-1}.

The main cultivation was carried out in a cylindrical 200-ml bubble column PBR (inner diameter 3.6 cm) sparged with 100 ml min^{-1} of 5%v/v CO_2-enriched air. Modified standard inorganic medium (MSIM) was used in this step, and its composition has been reported in detail elsewhere (15,16). Light intensity was kept unchanged and temperatures were varied at 20, 23.5, 27, and 30.5°C, by means of a thermostatic bath. During the growth stage, *H. pluvialis* cultures consume enormous amounts of nutrients, particularly N and P, which soon becomes a limiting factor. To avoid the onset of astaxanthin accumulation, nutrients were added to the culture using the MSIM stock solutions after the determination of the total nitrate concentration.

The astaxanthin accumulation experiments were carried out in the same 200-ml PBR where fully grown *H. pluvialis* cultures were centrifuged and resuspended at a final concentration of 1.0×10^6 cells ml^{-1} in the nitrogen deficient formulation of the MSIM medium (MSIM-N). In this medium the nitrates were substituted by equimolar concentrations of corresponding chloride salts. The temperatures investigated during the astaxanthin accumulation experiments were 20°C and 27°C.

Analytical procedures Dry weight (DW) was measured by filtering known aliquots of culture on pre-weighted glass membrane filters (GF/C; Whatman, GE Healthcare Life Sciences, USA). The membranes were stored in an oven at 80°C overnight and weighed the next day. The total cell number was measured using a Coulter counter (CDA-500, Sysmex, Japan).

Chlorophylls (CHLs) and total carotenoids were measured with a spectrophotometer after 90% acetone extraction according to Lichtenthaler (17). After a first centrifugation step to remove the culture medium, the culture was resuspended in tubes with 1 ml of 90%v/v acetone and about 1 ml of glass beads for cell disruption. After sufficiently shaking the tubes, a spectrophotometer (UV-1700, Shimadzu, Japan) was used to read the resultant supernatant at wavelengths of 450, 630, 645, 663, and 750 nm.

Astaxanthin was quantified spectrophotometrically according to Tolasa et al. (18) using acetone instead of *n*-hexane for the extraction.

Nitrate concentration was measured by spectrophotometric determination according to Armstrong (19). The culture supernatant (150 μl) was acidified with 100 μl of 1 M HCl, and brought to a level of 5 ml using distilled water. Sample absorbance was read at 220 nm against a distilled water blank, and the concentration was calculated using a predetermined calibration equation.

The oxygen evolution rate (OER) was measured in order to quantify the photosynthetic efficiency of *H. pluvialis* under different temperature conditions and draw the photosynthesis-intensity (PI) curve. The sample collected from an exponentially growing culture was diluted to 5 mg L^{-1} of CHL and illuminated by red light-emitting diode (LED) lights with variable intensity. The resultant OER was measured with an oxygen sensor (OE-8250M, DKK-TOA, Japan) with a current transmitted across a 10 kΩ resistor and read on a digital multimeter (AD7461A, Advantest, Japan) (20). The temperature was kept constant throughout the entire experiment (20°C and 27°C), and the calibration of the electrode was carried out against oxygen/nitrogen mixtures of known concentrations.

TABLE 1. Maximum cell concentration and maximum specific growth rate as a function of culture temperature.

Cultivation temperature (°C)	Maximum cell concentration (cells·ml^{-1})	Maximum specific growth rate (h^{-1})
20	1.67×10^6	0.0256
23.5	1.28×10^6	0.0295
27	1.27×10^6	0.0318
30.5	6.04×10^5	0.0335

RESULTS AND DISCUSSION

Cell number and DW The growth curves of *H. pluvialis* under temperatures of 20°C, 23.5°C, 27°C, and 30.5°C are shown in Fig. 1A. The culture kept at the lowest temperature (20°C) showed the highest growth in terms of cell number, which was the maximum cell concentration of about 1.7×10^6 cells ml^{-1}. Lower final concentrations were attained at the other temperatures. At temperatures of 23.5°C and 27°C, roughly the same concentration was reached at 1.3×10^6 cells ml^{-1}. At a temperature of 30.5°C, cell concentration ended at approximately 6.0×10^5 cells ml^{-1}, which was about 35% of the cell concentration attained in the culture grown at 20°C (Table 1). These results were similar to those obtained by Tjahjono et al. (12) and not surprising as the optimum temperature for this strain has been established at 20°C by the NIES. It was interesting to note, however, that *H. pluvialis* grown under temperature conditions at 23.5°C and 27°C was still capable of sustained growth showing no negative effect with the noted exception of a slightly lower final cell concentration. In fact, *Haematococcus* sp. strains isolated from different regions have shown the ability to actively grow at 25°C with no apparent problems (21,22). Moreover, the same strain as we used in the present study has shown similar growth performance under analogous cultivation conditions at 28°C (23).

When the maximum culture growth rate is considered to be a function of temperature, the opposite trend can be seen with respect to the maximum cell number (Table 1). The higher the

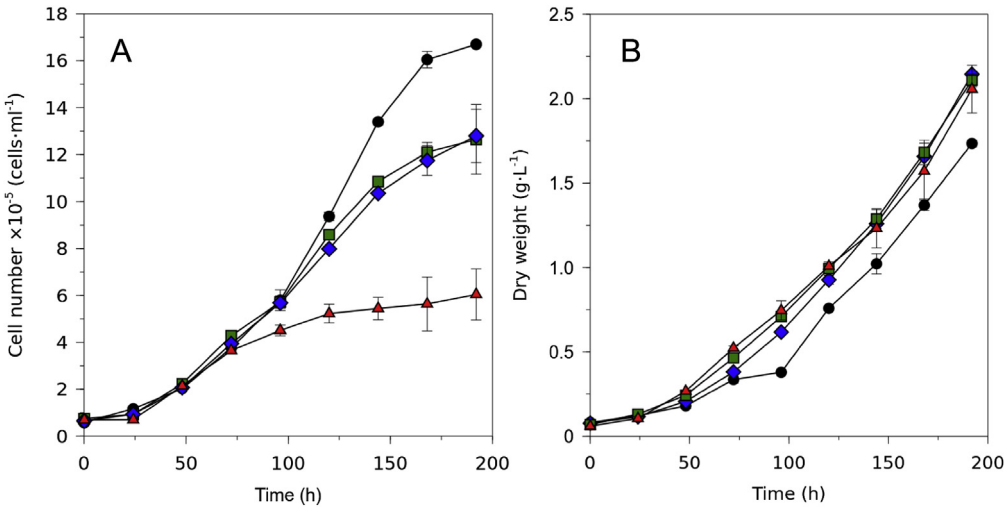

FIG. 1. Growth curves of *Haematococcus pluvialis* cultures at 20°C (circles), 23.5°C (diamonds), 27°C (squares) and 30.5°C (triangles): (A) culture cell number; (B) dry weight. Each plot represents the mean ± SD obtained from three different experiments.