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Effect of red cyst cell inoculation and iron(II) supplementation on autotrophic astaxanthin production by *Haematococcus pluvialis* under outdoor summer conditions

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ABSTRACT

The negative effect of heat stress on the autotrophic astaxanthin production by *Haematococcus pluvialis* has been observed during outdoor culture in summer. Under the summer conditions, the proliferation of vegetative cells was highly halted in the green stage and the inducibility in the biosynthesis of astaxanthin was partly hindered in the red stage. Herein, under outdoor summer conditions in which variations of the diurnal temperature occur, heat-stress-driven inefficient vegetative growth of *H. pluvialis* was highly improved by inoculating the red cyst cells; thereby, maintaining relatively moderate intracellular carotenoid levels in the green stage. Subsequently, a remarkably enhanced astaxanthin titer was successfully obtained by supplementing 50 μM iron(II) to induce the heat stress-driven Haber–Weiss reaction in the red stage. As a result, the productivity of astaxanthin in the cells cultured under summer temperature conditions (23.4–33.5 °C) using the two methods of red cell (cyst) inoculation and the iron(Fe^{2+}) supplementation was increased by 147% up to 5.53 mg/L day compared with that of the cells cultured under spring temperature conditions (17.5–27.3 °C). Our technical solutions will definitely improve the annual natural astaxanthin productivity in *H. pluvialis* in locations confronted by hot summer weather, particularly in large-scale closed photobioreactor systems.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), the highest value ketocarotenoid, has widespread applications in food supplement, nutraceutical, cosmetics and feed (Lorenz and Cysewski, 2000; Guerin et al., 2003). Among the various astaxanthin-accumulating species in nature, the green microalga *Haematococcus pluvialis* is the richest source of natural astaxanthin (up to 4% of its dry mass) (Boussiba, 2000) and is cultivated on an industrial scale (Bubrick, 1991; Cysewski and Lorenz, 2004; Li et al., 2011).

Industrial production of astaxanthin from *H. pluvialis* is based on a two-stage process to achieve a maximal astaxanthin titer since the optimal conditions for vegetative cell growth (low stress) differ from the conditions under which astaxanthin is synthesized (high stress) (Boussiba, 2000). In the first ("green") stage, two different green cells of flagellate (motile type) and palmelloid (non-motile type) increase *via* cell multiplication (vegetative growth). In the second ("red") stage, the cell proliferation is halted, but the single-cell mass begins to increase due to the morphological transformation from the green cells to red cyst cells to accumulate astaxanthin (Fig. 1A) (Fábregas et al., 2003).

Although heterotrophic culture with acetate is easy to obtain a high cell density (green stage) and high astaxanthin productivity (red stage) in *H. pluvialis*, autotrophic culture using CO_2 is relatively feasible for industrial production of astaxanthin in a large-scale outdoor culture process (Bubrick, 1991; Kaewpintong et al., 2007; Li et al., 2011). First, the use of acetate, which must be synthesized, increases the expense associated with maintenance of the system. Second, acetate, when added to a culture medium, easily evokes severe contamination with heterotrophic microorganisms like bacteria. Thirdly, astaxanthin accumulation is relatively low in heterotrophic process despite of high productivity compared to autotrophic process (Kobayashi et al., 1997).

Solar energy which radiates both light and heat can be directly used to reduce lighting costs. During the summer periods, an intense solar radiation commonly increases the ambient (air) temperature and the humidity. However, the high sensitivity of the

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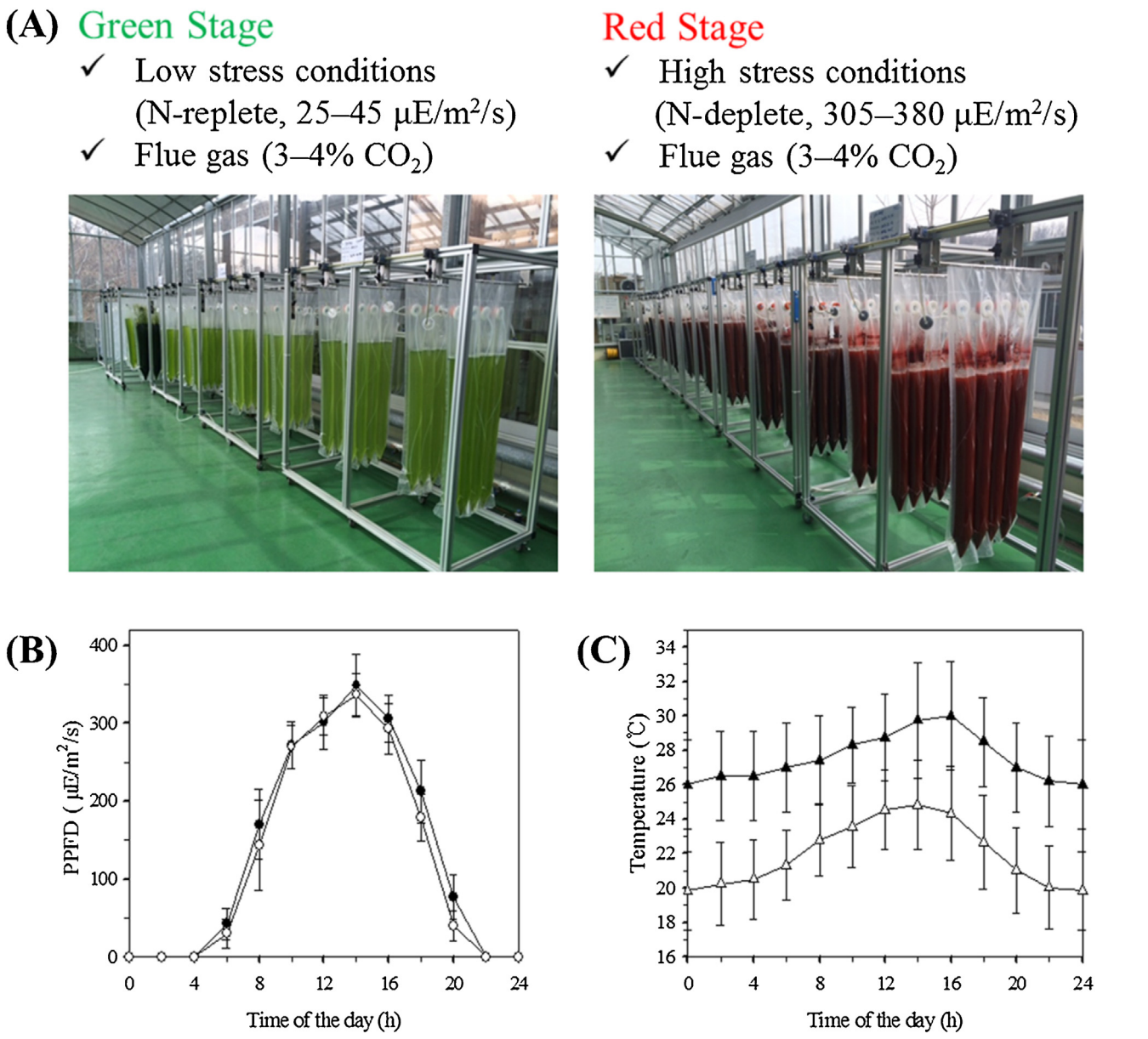

Fig. 1. (A) Photographic demonstration of the photoautotrophic *Haematococcus* culture system for outdoor production of astaxanthin *via* a two-stage strategy. (B) Photosynthetic photon flux density (PPFD) in spring (late March–late June) (○) and summer (late June–late September) (●) and (C) medium temperature in spring (△) and summer (▲) during the daylight period for *H. pluvialis* cultures. The data are given as the mean ± standard deviation of ninety diurnal measurements from three individual thin-film columns. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

green stage and the inefficient inducibility of the red stage to high temperature (HT) may hinder efficient astaxanthin production by *H. pluvialis* under outdoor summer conditions. In particular, when using a closed system like photobioreactor (PBR) during the summer months such problems are inevitably faced.

Under ambient (air) HTs, closed PBRs generally evoke a relatively rapid increase in the culture medium temperature compared to open ponds because the unit volume of the PBR is smaller than that of the pond. Nonetheless, closed PBR system is generally easier to achieve the high efficiency in biomass production and to control contamination than those of open pond system.

It has been reported recently that HTs (30–36 °C) that exceed 28 °C evoke inefficient induction that diminishes both cyst growth and astaxanthin production under photoautotrophic conditions (Giannelli et al., 2014; Wan et al., 2014; Hong et al., 2015). Although the iron(II) supplementation method greatly improved the inefficient induction under indoor conditions of constant exposure to continuous HTs (30–36 °C), the actual applicability of the developed technique has not yet been proven in outdoor summer conditions in which variations of the diurnal temperature occur.

Herein, we report for the first time that a closed *Haematococcus* culture system was successfully operated in the PBR using two methods of red cyst inoculation for the green stage and 50-μM Fe^{2+} supplementation for the red stage under outdoor summer conditions. These results clearly indicate that continuous maintenance of relatively moderate intracellular carotenoid levels during photoautotrophic culture is a notably crucial factor for astaxanthin production by *H. pluvialis* at elevated temperatures.