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Effect of initial biomass density on growth and astaxanthin production of *Haematococcus pluvialis* in an outdoor photobioreactor

Junfeng Wang · Danxiang Han · Milton R. Sommerfeld · Congming Lu · Qiang Hu

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Abstract Initial biomass density (IBD) is an important factor that affects the viability and productivity of microalgae particularly when sunlight is used for photosynthesis. In this paper, the effect of IBD on photosynthesis, growth, and astaxanthin production of the green microalga *Haematococcus pluvialis* during the astaxanthin induction stage was studied in a glass column photobioreactor during different seasons. Of seven IBDs, i.e., 0.1, 0.5, 0.8, 1.5, 2.7, 3.5, and 5.0 g L^{-1} tested, 0.8 g L^{-1} IBD was optimal and resulted in the highest astaxanthin productivity of 17.1 mg L^{-1} day^{-1}. Severe photoinhibition of photosynthesis occurred at low IBD (e.g., 0.1 g L^{-1}) cultures, especially in the winter, and severe light limitation to individual cells in high IBD cultures (>2.7 g L^{-1}) were responsible for reduced astaxanthin production. This was the first report quantitatively assessing IBD as the key limiting factor for astaxanthin production in *H. pluvialis* outdoor cultivation.

Keywords *Haematococcus pluvialis* · Astaxanthin · Initial biomass density · Photoinhibition · Photobioreactor

J. Wang · C. Lu
Photosynthesis Research Center, Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, People's Republic of China

J. Wang · D. Han · M. R. Sommerfeld · Q. Hu (✉)
Department of Applied Sciences and Mathematics, Arizona State University, Polytechnic Campus, 7001 E. Williams Field Road, Mesa, AZ 85212, USA
e-mail: huqiang@asu.edu

Introduction

Astaxanthin is a keto-carotenoid responsible for the pinkish color of some fish, crustaceans, and birds and is used as a pigmentation agent in aquaculture and as a potent antioxidant for human health (Benemann 1992; Goswami et al. 2010; Guerin et al. 2003). The green microalga *Haematococcus pluvialis* is the best known source of natural astaxanthin (Margalith 1999). A common strategy for the production of astaxanthin in *H. pluvialis* is to sustain rapid growth under favorable culture conditions (e.g., low light and nutrient repletion) ("green stage") and then stress the cells by introducing high light and/or nutrient depletion to induce astaxanthin production ("red stage"). Although this "two-stage" batch mode has been adopted by the *Haematococcus* industry and research community (Aflalo et al. 2007; Fábregas et al. 2001; Lu et al. 1994; Sarada et al. 2002), a persisting, largely ignored problem associated with this common practice is that considerable cell death occurs upon transferring *H. pluvialis* from the green stage to the red stage. The cell mortality rate may range from 20 to 80 % of total cells subjected to stress, depending on strains, bioreactors, and stress conditions under which *H. pluvialis* cells are maintained (Harker et al. 1996; Kobayashi et al. 1997; Hu et al. 2008; Li et al. 2010). As a result, overall astaxanthin productivity in *H. pluvialis* cultures is low. For example, an average astaxanthin production rate of 2.2 mg L^{-1} day^{-1} was obtained from the outdoor cultivation of *H. pluvialis* in a 75,000-L photobioreactor (Olaizola 2000).

High light or high light in combination with nutrient depletion are the major factors that stimulate astaxanthin synthesis in *H. pluvialis* in a photoautotrophic culture mode, but they may also cause severe photoinhibition of photosynthesis or photooxidative damage of the cells (Wang et al. 2009; Hu et al. 2008; Li et al. 2008, 2010). It is imperative

that the intensity of incident light provided to the culture must be such that it maximizes astaxanthin production while minimizing its ill-effect on cell mortality.

We hypothesized that an optimal incident light intensity for maximum astaxanthin productivity can be created by starting the red stage culture with a proper initial biomass density (IBD). Here, we provide a detailed study of the effects of IBD on growth and astaxanthin accumulation of *H. pluvialis* at the red stage in an outdoor glass column photobioreactor. The photochemical efficiency of photosynthesis, specific growth rate, pigment content, and biomass concentration were monitored as a function of IBD ranging from 0.1 to 5.0 g L^{-1} of cell dry weight during different seasons, i.e., summer, fall, and winter.

Materials and methods

Haematococcus pluvialis Flotow K-0084 was obtained from the Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen, Denmark and maintained in BG11 culture medium (Boussiba and Vonshak 1991). In the green stage, *H. pluvialis* was cultivated in a glass column (inner diameter 5 cm; working volume 0.6 L) under indoor conditions (20 °C, 20 μmol photons m^{-2} s^{-1} of continuous light).

Red stage experiments were conducted in glass columns of the same specifications. Sixteen columns were placed in two transparent plastic racks arranged side by side on an east-western orientation. Culture mixing was provided by aeration (1.5 % CO_2, *v/v*) through a glass capillary tube inserted near the bottom of the column. The culture temperature was maintained at 20–25 °C during the daylight period by automatically spraying prechilled water onto the surface of the columns. The minimum culture temperature was not regulated. The photosynthetic photon flux density impinging on the bioreactor surface was determined with a Li-190SB quantum sensor coupled to a Li-250A data logger (Li-Cor, USA). Culture temperature was measured with a digital long-stem thermometer equipped with a stainless steel probe (VWR International, USA). Experiments were carried out at the outdoor algae research facilities at Arizona State University Polytechnic campus in Mesa, Arizona (33°24′ N, 111°49′ W, Mesa, USA) from July to December 2008. In this paper, September, October, and December are referred to as summer, fall, and winter, respectively.

Biomass concentration was measured by a gravimetric method (Li et al. 2010). The samples were collected at 7:00–8:00 p.m. after the sunset. The specific growth rate (μ, day^{-1}) was calculated as follows:

$$\mu = [\ln(DW_t) - \ln(DW_0)]/t \quad (1)$$

Biomass productivity (g L^{-1} day^{-1}) was calculated as follows:

$$\text{Biomass productivity} = (DW_t - DW_0)/t, \quad (2)$$

in which the DW_t and DW_0 were the biomass of day t and day 0, respectively.

Ten mg of freeze-dried *H. pluvialis* sample was ground by a prechilled mortar and pestle in liquid nitrogen and then the cell-free powder was extracted in a small amount of solvent solution (methanol/dichloromethane=1:3, *v/v*). The sample was centrifuged at 10,000×*g* for 10 min. The pellet was then extracted several times to colorless with the solvent solution. The supernatants were combined and dried with compressed nitrogen gas. The entire procedure was carried out under dim light. Extracted pigment sample was redissolved in the solvent solution to determine astaxanthin concentration by HPLC (Waters, USA) following the protocol reported by Yuan et al. (2002), and the chlorophyll concentration was measured using a spectrophotometer according to Lichtenthaler and Wellburn (1983).

The nitrate (NO_3^-) concentration of culture was measured by Quikchem 8500 (Lachat, USA). The maximum photochemical efficiency of photosystem II (F_v/F_m) is a chlorophyll fluorescence parameter that reflects the photoinhibitory status of the reaction centers of photosystem II (PSII). The daylight variation of F_v/F_m was determined hourly with Dual-PAM 100 (Walz, Germany). Algal samples were dark-adapted for 15 min before measurements. One mL of sample was loaded into the cuvette and mildly stirred with a magnetic stir bar. The measuring light was turned on, and signal intensity was maintained between 0.1 and 0.2 V. The recording program was run for 5–10 s before a high intensity pulse light (10,000 μmol photons m^{-2} s^{-1}, lasting 0.8 s) was applied to fully closed PSII reaction centers (Genty et al. 1989). The minimum and maximum fluorescence, F_o and F_m, were recorded automatically during the procedure. The maximum photochemical efficiency of PSII was calculated as follows: $F_v/F_m = (F_m - F_o)/F_m$

Results

The effects of IBD on cell mortality, morphology, growth, pigment content, and productivity of biomass and astaxanthin of *H. pluvialis* at the red stage was studied in the outdoor glass columns, and the results are shown in Fig. 1. The cultures with 0.1 g L^{-1} IBD exhibited minimum biomass accumulation (Fig. 1d). Considerable cell bleaching (or death) and astaxanthin formation in the living cells were observed within 2 days (Fig. 1a). With increase in IBD from 0.1 to 1.5 g L^{-1}, less cell mortality but greatly increased biomass concentrations were observed, though less red pigmentation occurred in the cells

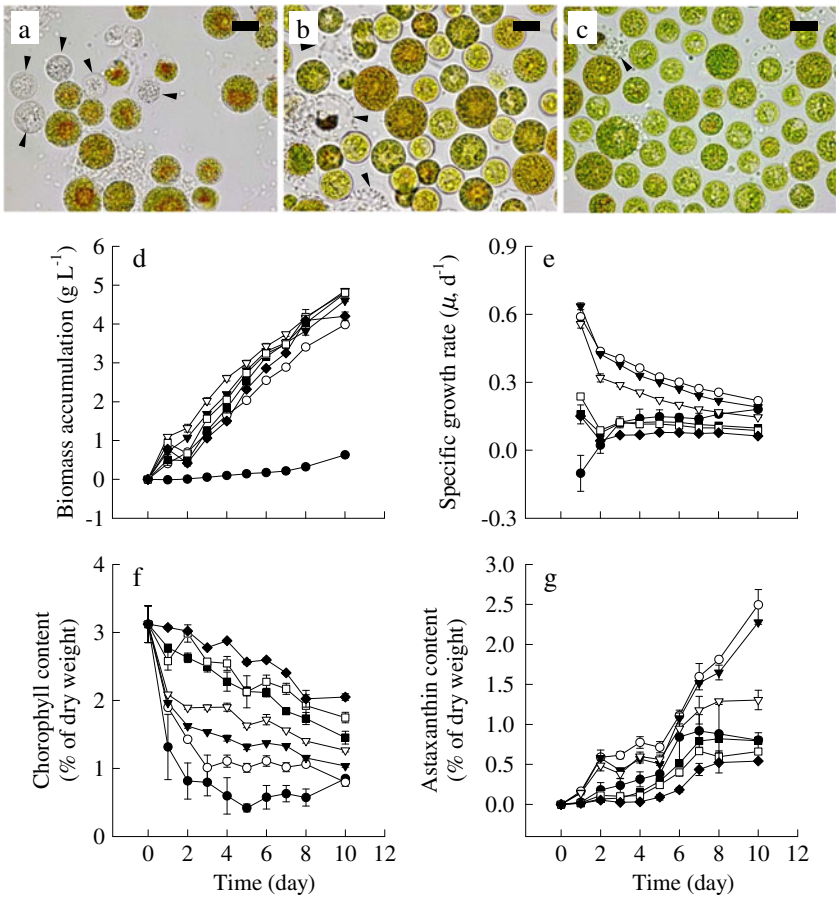

Fig. 1 Effect of different initial cell densities on cell morphology and viability (**a**–**c**), biomass accumulation (**d**), specific growth rate (**e**), and chlorophyll (**f**) and astaxanthin contents (**g**) of *H. pluvialis* grown in glass columns outdoors. The IBDs were 0.1 (*black circle*), 0.5 (*white circle*), 0.8 (*black triangle*), 1.5 (*white triangle*), 2.7 (*black square*), 3.5 (*white square*), and 5 (*diamond*) g L^{-1}. Light microscopic images were 2-day stressed *H. pluvialis* cells in 0.1 (**a**), 1.5 (**b**), and 5.0 (**c**) g L^{-1} IBD cultures. *Bar*=20 μm. The bleached cells are indicated by *arrows*. Data are mean ± standard deviation of six measurements from three individual glass columns. The biomass accumulation (**d**) are the differences of cell dry biomass measured on day *x* and day 0, where *x*=day 1, 2, 3, etc

(Fig. 1b, d). Further increasing IBD from 1.5 to 5.0 g L^{-1} led to slight decrease of net biomass accumulation and the cells remained green, but cell mortality was greatly reduced (Fig. 1c, d). The maximum specific growth rate (μ) of 0.6 day^{-1} was obtained in 0.1, 0.5, 0.8, and 1.5 g L^{-1} IBD cultures, which was the highest reported in outdoor culture of *H. pluvialis* (Fig. 1e and Table 1). The higher performance of these cultures was in part due to the availability of nitrogen during 10 days of cultivation.

A decrease in chlorophyll concentration was observed in all the IBD cultures and a general trend was that the lower the IBD the more rapid decline in chlorophyll occurred. However, the kinetics of chlorophyll reduction was different. When the IBD was 1.5 g L^{-1} or lower, a two-phase response was evident: a rapid decrease of chlorophyll occurred within the first day or two, followed by either a slow, gradual decrease in chlorophyll or chlorophyll remaining at a more or less constant level. When the IBD was 2.7 g L^{-1} or higher, the chlorophyll content decreased in a linear fashion (Fig. 1f). No astaxanthin was detected in the cells at the green stage stage. Astaxanthin biosynthesis was induced in the red stage culture outdoors. However, both the rate of astaxanthin accumulation and the maximum cellular astaxanthin content in the cells of different IBDs were different. At 0.1 g L^{-1} IBD, the maximum astaxanthin content was ca. 0.6 % on a per dry weight (dwt) basis (Fig. 1g). The maximum astaxanthin content of ca. 2.5 % dwt occurred in the 0.5 g L^{-1} IBD cultures. When the IBD increased further, the maximum astaxanthin content declined, i.e., the higher the IBD, the lower the rate of astaxanthin accumulation and the lower the maximum astaxanthin content.

Figure 2 shows the changes in nitrate concentration in the different IBD cultures. Rapid uptake of nitrate occurred in these cultures during the first 3–4 days, corresponding to the high specific growth rates observed during this time period. The nitrogen update rates reduced gradually thereafter, and ca. 4 mM nitrate remained in the cultures at the end of the 10-day cultivation.

The maximum biomass productivity of 0.1 g L^{-1} IBD cultures was less than 0.1 g L^{-1} day^{-1} at the red stage for 10 days (Fig. 3a). A 4- to 6-fold increase in the maximum biomass productivity was obtained when IBD was 0.5 g L^{-1} or greater. The biomass productivity in 0.5, 0.8, 1.5, 2.7, and 3.5 g L^{-1} IBD cultures reached maximum levels after 4 days of cultivation. However, the maximum biomass productivity in the 5.0-g L^{-1} IBD cultures did not occur until 8 days. Once the maximum biomass productivity occurred, the value either declined (e.g., in the 0.8 and 1.5 g L^{-1} IBD cultures), or remained more or less stable (e.g., in the 0.5,

Table 1 Comparison of the growth and astaxanthin production of *H. pluvialis* under outdoor conditions

Strain	Culture apparatus	Stress at red stage	Culture Model	Red stage IBD (g L^{-1})	Red stage overall specific growth rate (day^{-1})	Astaxanthin content (% dwt)	Red stage astaxanthin productivity (mg L^{-1} day^{-1})	Red stage biomass productivity (g L^{-1} day^{-1})	References
AQSE002	Tube, pond	N/A	Batch	N/A	<0.175[a]	3.4	N/A	2.20[a]	Olaizola 2000
CCAP 34/8	Tube, column	Nitrogen starvation	Batch	0.4	0.18[a]	1.1	4.8[a]	0.55	López et al. 2006
Local isolation	Tube	Nitrogen starvation	Batch	0.6	0.07[a]	3.6[a]	7.2[a]	0.05[a]	Torzillo et al. 2003
K-0084	Column, panel, tube	High light, nutrient deprivation	Batch	1.5	0.15[a]	3.8	<14.8	0.21	Aflalo et al. 2007
CCAP 34/8	Tube	High light	Continuous	N/A	N/A	1.3	8.0	0.01[a]	García-Malea et al. 2009
H. pluvialis 26	Open pond	High light	Batch	N/A	N/A	2.8	4.3[a]	<0.15	Zhang et al. 2009
K-0084	Column	High light	Batch	0.8	0.23	2.7	17.1	0.58	Wang et al. this work

[a] Recalculated value by the authors

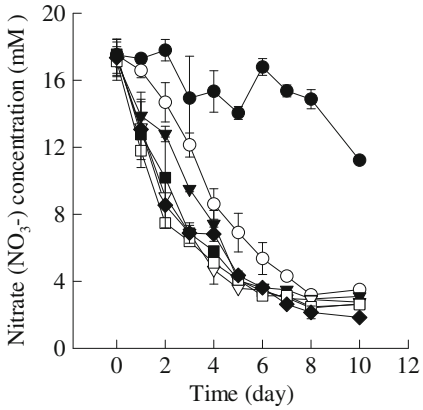

Fig. 2 Nitrate uptake kinetics as affected by initial biomass density in *H. pluvialis* cultures. The IBDs were 0.1 (*black circle*), 0.5 (*white circle*○), 0.8 (*black triangle*), 1.5 (*white triangle*), 2.7 (*black square*), 3.5 (*white square*), and 5 (*diamond*) g L^{-1}. Data are mean ± standard deviation of six measurements from three individual glass columns

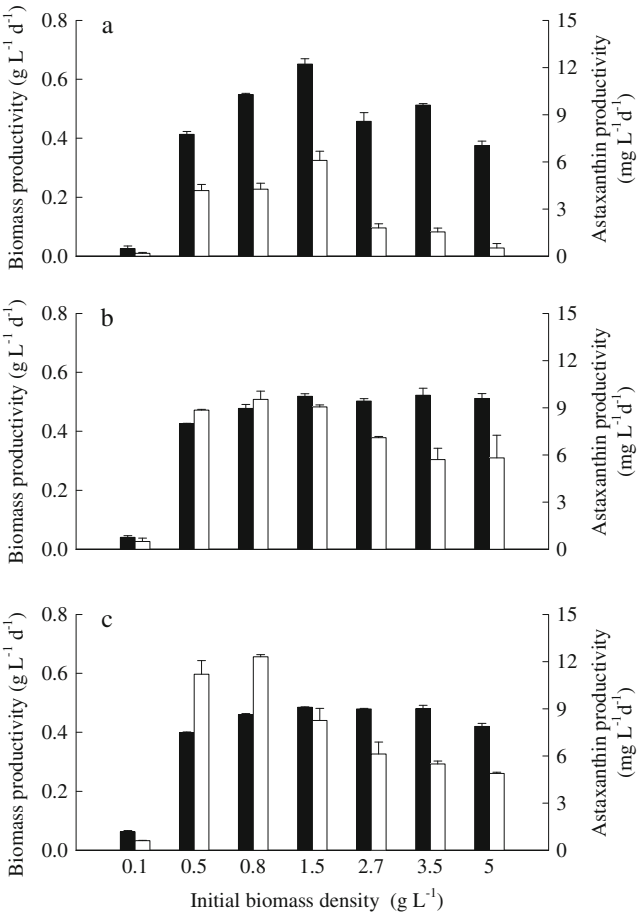

Fig. 3 Effect of initial cell density on red stage biomass and astaxanthin productivities of *H. pluvialis* grown in glass columns outdoors. **a**–**c** Biomass productivity (*closed bars*) and astaxanthin productivity (*open bars*) at days 4, 8, and 10 of cultures of the different IBDs, respectively. The data are mean ± standard deviation of six measurements from three individual glass columns

2.7, 3.5, and 5.0 g L^{-1} IBD cultures) in 10 days of red stage cultivation (Fig. 3b, c) The lowest IBD cultures led to the lowest astaxanthin productivity. When the IBD increased to 0.8 g L^{-1}, astaxanthin productivity reached the maximum value of 13.5 mg L^{-1} day^{-1} after 10 days of cultivation. Further increasing IBD above this level resulted in decreasing astaxanthin productivity (Fig. 3).

In order to determine how seasonal changes in light intensity and temperature would affect the relationship between IBD and growth and astaxanthin production in *H. pluvialis* cultures, we selected 0.1, 0.8, and 5.0 g L^{-1} IBDs, representing a low, medium, and high IBD, respectively, to conduct a series of outdoor experiments from September (summer) through December (winter) 2008. Figure 4c, d shows hourly solar radiation and culture temperatures measured on a typical day in October (fall) and December (winter) when the experiments were conducted. The amount of solar radiation impinging on the surface of the columns during the daylight period followed a two-peak pattern, one measured between 8:00–9:00 a.m. and the other between 3:00–4:00 p.m. The maximum hourly solar radiation of ca. 2,000 μmol photons m^{-2} s^{-1} was measured on the surface of the columns both in October and December, though the daylight period was shorter by ca. 1 h in December than in October (Fig. 4c). The culture temperature was usually between 0 and 5 °C in the morning (6:00 a.m.) during the winter (December), and it increased steadily to reach 18–20 °C by 10:00–11:00 a.m. and remained at that level throughout the daylight period (Fig. 4d).

Figure 5a–c depicts the growth curves of *H. pluvialis* cultures of three different IBDs in summer, fall, and winter. At 0.1 g L^{-1} IBD cultures, no growth was observed in summer and winter. The cells were photobleached within the first day. A small, but noticeable, growth occurred in the fall after a long lag phase (Fig. 5a). At the medium IBD, a linear growth was observed in *H. pluvialis* cultures in all the seasons investigated. The growth gradually increased from winter to summer due to the gradual increase of solar radiation and the improved culture temperatures during daylight (Figs. 4c, d and 5b). At the high IBD, the trend of growth followed essentially the same pattern as observed in the medium IBD cultures but at much reduced μ (Fig. 5c). The maximum astaxanthin content in the low IBD culture was 2.0±0.1 % in fall (Fig. 5d). It reached 2.7±0.1 % in the medium IBD cultures in the summer and fall cultures (Fig. 5e). In the high IBD cultures, the maximum astaxanthin

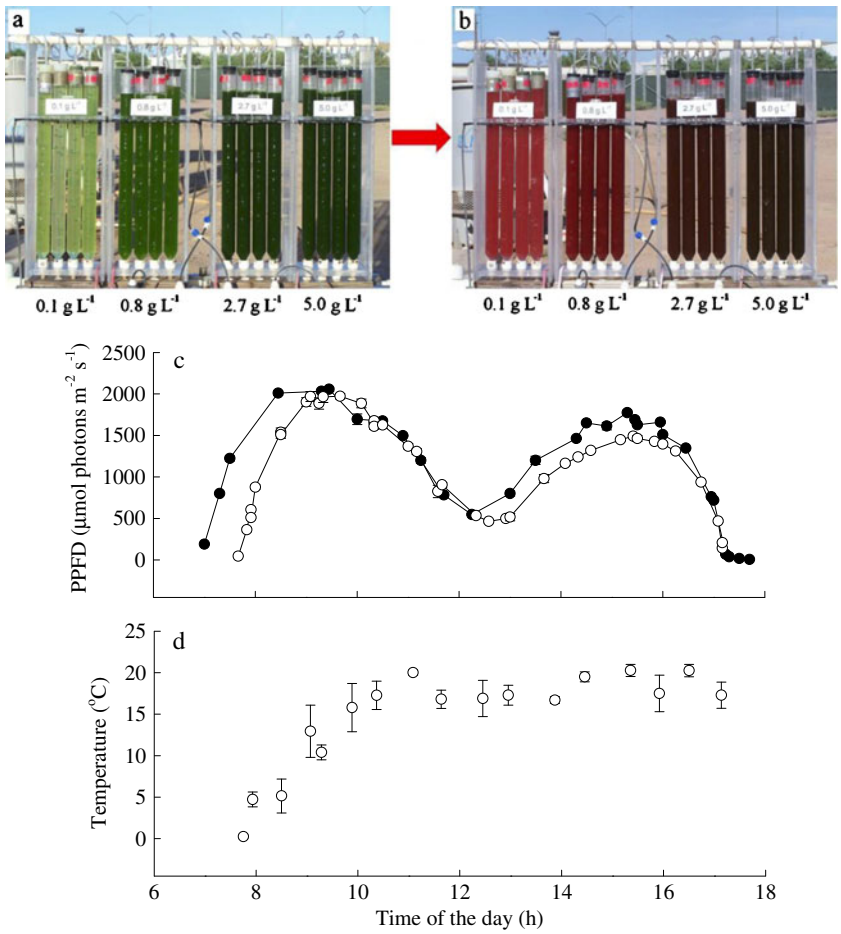

Fig. 4 The green stage (**a**) and red stage (**b**) cultures of *H. pluvialis* in glass columns outdoors, photon flux density (**c**), and temperature (**d**) during the daylight period of *H. pluvialis* cultures in a typical day in the fall (*black circle*) and winter (*white circle*). The data are mean ± standard deviation of five to ten measurements from three individual glass columns

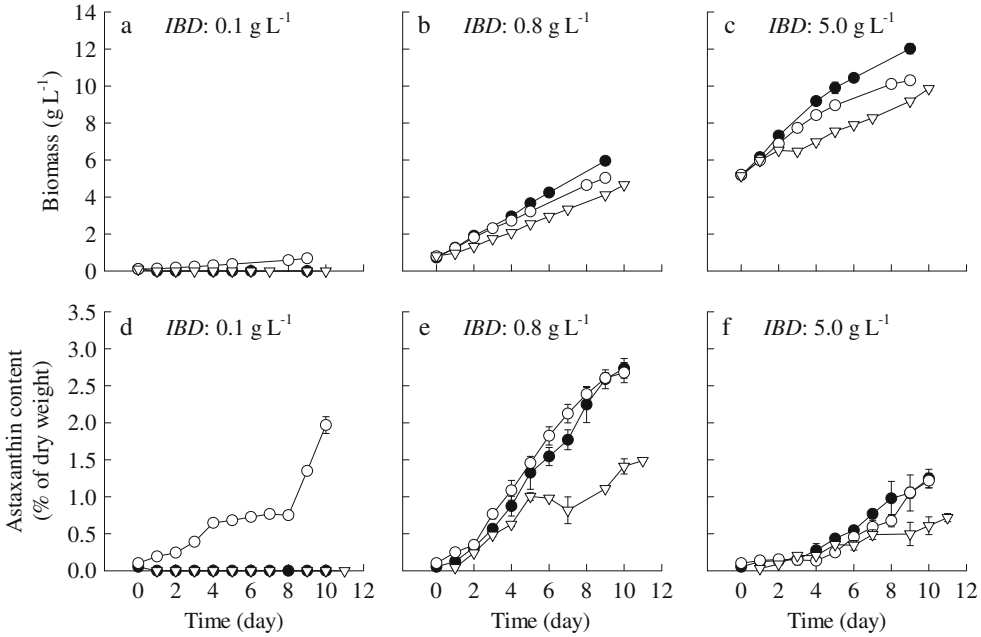

Fig. 5 The growth and astaxanthin content of *H. pluvialis* cultures of the different IBDs in the summer (*black circle*), fall (*white circle*), and winter (*white triangle*). The data are mean ± standard deviation of six measurements from three individual glass columns

content was 1.2±0.1 % in the summer and fall and decreased to 0.7±0.05 % in the winter (Fig. 5f).

In order to determine how photosynthesis was affected by IBD in the different seasons, we measured the maximum photochemical efficiency (F_v/F_m), a chlorophyll fluorescence parameter that reflects the photoinhibition status of the reaction centers of photosystem II. The value of F_v/F_m is generally between 0.78–0.84 when algae cells are healthy, and it decreases under stress (Stirbet and Govindjee 2011). Figure 6 shows the daylight change of F_v/F_m in the low, medium, and high IBD cultures in fall and winter. A F_v/F_m of 0.82±0.03 was measured right after being transferred outdoors, indicative of the maximum photosynthetic efficiency of photosynthesis the green stage cells possessed. In the fall, the F_v/F_m of the low IBD cultures declined to ca. 0.3 during the morning hours on the first day, indicative of severe photoinhibition of photosynthesis. As the cultures proceeded, the cells gradually recovered from photoinhibition (Fig. 6a–c). In the winter, the F_v/F_m in the low IBD cultures dropped from the maximum of 0.83±0.02 to below the detection level within the first few hours of the first day and did not recover due to low temperature-induced photooxidative damage of the cells (Fig. 6d–f). In the fall, the F_v/F_m of the medium IBD cultures decreased gradually during the morning

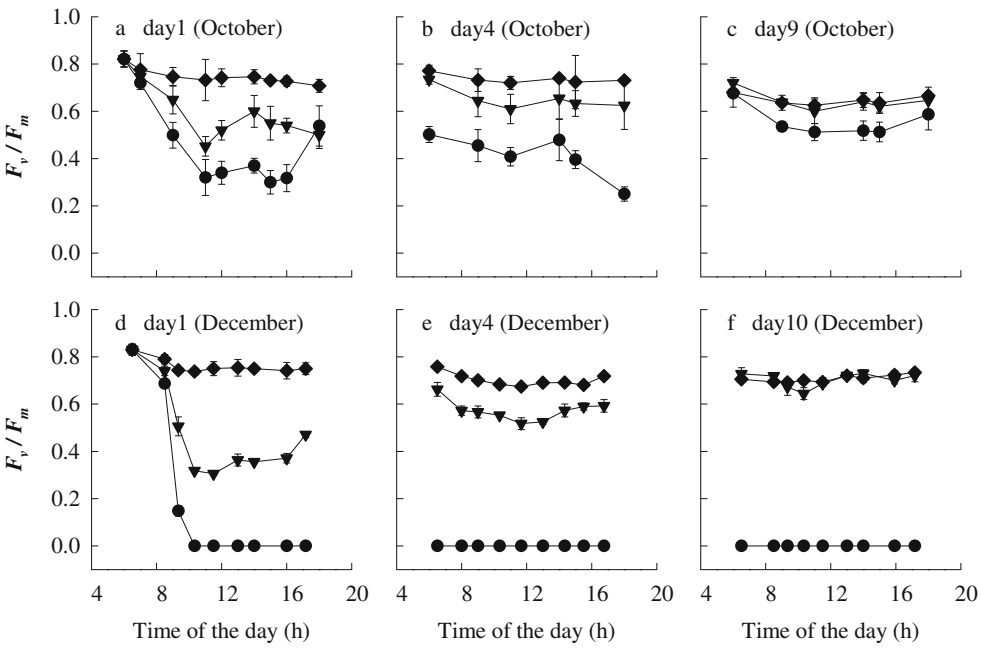

Fig. 6 Daylight changes on the maximum photochemical efficiency of *H. pluvialis* with different IBDs in the fall (October) and winter (December). The data are mean ± standard deviation of six measurements from three individual glass columns. The IBDs are 0.1 (*black circle*), 0.8 (*black triangle*), and 5.0 g L^{-1} (*diamond*)

hours and recovered to some extent on the afternoon of the first day. It varied to a lesser extent on day 4 and showed little change on day 9. In winter, the medium IBD cultures followed the similar trend to that observed in the fall, but the cells experienced somewhat deeper decline in F_v/F_m during the daylight period, indicative of more severe low temperature-induced photoinhibition on the first day of the red stage cultivation in the winter than in the fall. The high IBD cultures exhibited little daylight variation in F_v/F_m in the fall and winter, suggesting that high IBD can prevent not only high light-induced but also low temperature-induced photoinhibition of photosynthesis.

The biomass productivities of the low (0.1 g L^{-1}), medium (0.8 g L^{-1}), and high IBD (5.0 g L^{-1}) cultures in the different seasons are shown in Fig. 7a–c. In the medium and high IBD cultures, the maximum biomass productivities were achieved in the summer, whereas that of the low IBD cultures occurred in the fall with no productivity in the summer and winter (Fig. 7a–c). The maximum red stage astaxanthin productivities of 3.9, 17.1, and 14.0 mg L^{-1} day^{-1} were obtained in the low, medium, and high IBD cultures, respectively (Fig. 7d–f). The astaxanthin productivity of 17.1 mg L^{-1} day^{-1} in the red stage was the highest figure reported for outdoor mass culture of *H. pluvialis* (Table 1). With a given IBD, a large variation in astaxanthin productivity in the different seasons was attributable to culture temperature and to a lesser extent, light availability in terms of light intensity and length of daylight period. When a 4-day green stage period was taken into consideration, the volumetric astaxanthin productivities of low, medium, and high IBD cultures were 2.2, 9.1, and 7.4 mg L^{-1} day^{-1}, respectively.

Discussion

The effect of the different IBDs on growth and astaxanthin content and thus productivity of biomass and astaxanthin in *H. pluvialis* culture may be attributable to its influence on light availability to individual algal cells in the culture. Due to mutual shading of cells in the culture, the frequency of light–dark cycle and total length of light period during which individual cells are exposed to incident light intensity depends on IBD, i.e., the higher the IBD, the higher the frequency of light–dark cycle and the shorter the light exposure time individual cells receive from each light–dark cycle.

Low growth or photobleaching of 0.1 g L^{-1} IBD cultures was clear evidence of photodamage of photosynthetic machineries in *H. pluvialis* cells by exposure of the diluted cultures to full sunlight. The highest specific growth rate and biomass productivity occurring in 1.5 g L^{-1} IBD cultures represented in effect the optimal frequency of light–dark cycle and the length of light exposure under the experimental conditions. Further increasing of IBD, however, may have created shorter frequencies of light–dark cycle and longer light exposure times than the optimum ranges, resulting in reduced growth and biomass production. Note that the optimum IBD (0.8 g L^{-1}) for maximum astaxanthin production was considerably lower than that (i.e., 1.5 g L^{-1}) for maximum biomass production. This was because astaxanthin synthesis is induced by a higher light intensity and longer light exposure time than that required for optimal growth.

Li et al. (2010) studied the effects of different incident light intensities (i.e., 50, 125, 300, and 600 μmol photons m^{-2} s^{-1}) on growth and astaxanthin synthesis in *H. pluvialis*.

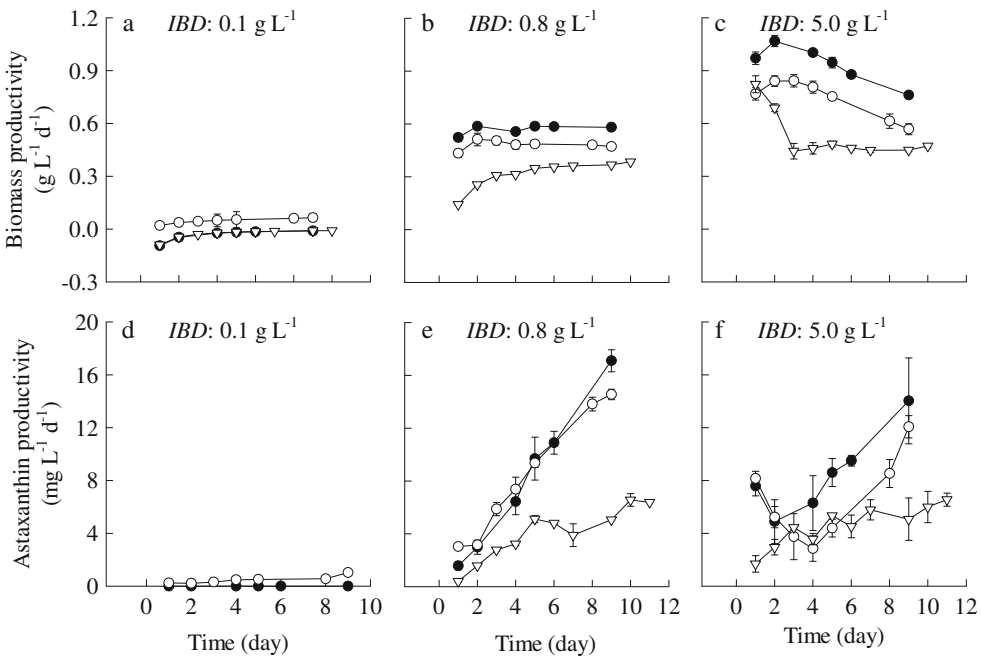

Fig. 7 The biomass and astaxanthin productivity of *H. pluvialis* cultures with the different IBDs in the summer (*black circle*), fall (*white circle*), and winter (*black triangle*). The data are mean ± standard deviation of six measurements from three individual glass columns

The results indicated that 300 μmol photons m^{-2} s^{-1} was the optimal light intensity for maximum astaxanthin production. A further increase in the light intensity to 600 μmol photons m^{-2} s^{-1} did not result in a higher astaxanthin content but resulted in more cell death. The maximum solar radiation in many geographical locations where mass culture of *H. pluvialis* occurs can be 2,000 μmol photons m^{-2} s^{-1} or greater that may potentially be an impediment to astaxanthin synthesis or cause cell death if incident light intensity is not reduced by shading of the cultures or increasing the light path of photobioreactors. A drawback of these approaches is reduction of potentially useful solar energy for maximum photosynthesis and thus overall astaxanthin production. Therefore, the identification and maintenance of an appropriate IBD represents an effective strategy to fully utilize solar radiation for sustaining maximal astaxanthin production while minimizing cell damage caused by excess solar radiation.

The astaxanthin content of 2.7 % dwt obtained from this study was considerably lower than that obtained in some of the previous outdoor cultures of *H. pluvialis*, e.g., 3.6 % dwt astaxanthin reported by Torzillo et al. (2003) and 3.8 % dwt astaxanthin reported by Aflalo et al. (2007). This was because the red stage culture was under nitrogen-replete conditions in this study (Fig. 2), whereas the red stage cultures in the previous studies were subjected to nitrogen-depleted conditions, which is known to be another stimulus that triggers astaxanthin production (Lu et al. 1994). In a separate investigation, we demonstrated that the depletion of nitrogen in our culture system also resulted in a higher astaxanthin content of ca. 3.8 % dwt (J. Wang, personal communication).

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