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An Alternative Approach to the Traditional Mixotrophic Cultures of *Haematococcus pluvialis* Flotow (Chlorophyceae)

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In traditional mixotrophic cultures of microalgae, all the inorganic nutrients and organic carbon sources are supplied in the medium before inoculation. In this study, however, an alternative approach was adopted in *Haematococcus pluvialis* Flotow, a microalga capable of growing mixotrophically on sodium acetate (Na-Ac). First, the cells were grown under 75 μmol photons m^{-2} s^{-1} phototrophically without Na-Ac until the stationary phase and then exposed to five different light regimes by the addition of Na-Ac (*e.g.*, dark, 20, 40, 75, and 150 μmol photons m^{-2} s^{-1}). Dry weight (DW), pigments, and especially cell number in alternative mixotrophy (AM) were higher than traditional mixotrophy (TM). Cell number in AM almost doubled up from 21.7 to 42.9×10^4 cells/ml during 5-day exposure to Na-Ac, whereas the increase was only 1.2-fold in TM. Maximum cell density was reached in 75 μmol photons m^{-2} s^{-1} among the light intensities tested. We propose that Na-Ac in TM of *H. pluvialis* can not be utilized as efficiently as in AM. With this respect, AM has several advantages against TM such as a much higher cell density in a batch culture period and minimized risk of contamination owing to the shorter exposure of cells to organic carbon sources. In consequence, this method may be used for other strains of the species, and even for the other microalgal species able to grow mixotrophically.

Keywords: *Haematococcus pluvialis*, vegetative growth, mixotrophic culture, acetate, pigments

The keto-carotenoid astaxanthin (AX), 3,3′-dihydroxy-β, β-carotene-4-4′-dione, especially the microbial one, is an important pigment exploited in various industries such as aquaculture, poultry, and health [4, 10, 13, 15, 31]. *Haematococcus pluvialis* Flotow, a freshwater microalga, is the most promising microbial producer of AX owing to the higher cellular content compared with the others (*i.e.*, more than 4% of dry weight) [18, 28].

In general, AX production from *H. pluvialis* is achieved through a two-stage culture: vegetative (green) and aplanospore (red) stages [1, 6]. In vegetative stage, the slow growth rate, low cell concentration, and susceptibility to contamination are the major problems [19]. In this respect, various studies were performed to improve the growth mainly on the optimization of culture medium [2, 7, 16, 23], light intensity [29], and organic carbon nutrition [9]. Although the use of Na-Ac as an organic carbon source under mixotrophic condition seems to be a favorable way of boosting cell concentration and growth rate in *H. pluvialis*, it also increases the contamination risk, particularly with bacteria and *Chlorella* sp., a green microalga able to grow very fast on organic substances.

In the present study, a new approach to use of Na-Ac in *H. pluvialis* cultures was investigated. In traditional mixotrophic algal cultures, an organic carbon source is present in the medium together with the other inorganic nutrients at the beginning of the culture. In our study, however, the cultures were initially grown phototrophically by inorganic nutrients, and Na-Ac addition was done subsequently at the end of the log phase under different light intensities. In this respect, the study aimed to show the effect of the new method against traditional mixotrophy (TM).

Materials and Methods

Organism and Culture Conditions

The unialgal culture of *Haematococcus pluvialis* Flotow (strain 5/99) was obtained from the culture collection of the CNR-Istituto per

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lo Studio degli Ecosistemi of Florence (Italy). The stock cultures were grown photoautotrophically in standard BG11 medium containing 1.5 g/l $NaNO_3$ [25]. All the trials were carried out in 1-l cylindrical glass bottles (10 cm in diameter). The cells were grown in batch cultures and aerated by bubbling air (1 vvm) under constant temperature at 25±0.5°C. The cultures in the first trial were illuminated continuously with daylight fluorescent lamps at a photon flux density (PFD) of 75 µmol photons m^{-2} s^{-1}. In the second trial, the cells were grown under 75 µmol photons m^{-2} s^{-1} until the stationary phase and then exposed to five different light regimes by the addition of Na-Ac (e.g., dark, 20, 40, 75, and 150 µmol photons m^{-2} s^{-1}). Photosynthetically active radiation (PAR) was measured with a LI-250A light-meter (Li-Cor, U.S.A.).

Application of Organic Carbon Source

Na-Ac was used as organic carbon source, and added in the culture medium in two ways: together with the other inorganic nutrients at the beginning of the culture as in traditional mixotrophy (TM), or at the end of the log phase in phototrophic mode as alternative mixotrophy (AM). Accordingly, the same concentrations of Na-Ac (1 g/l) were used in both trials. The trials were carried out in triplicate.

Analytical Procedures

For dry weight (DW) measurements, a 25-ml sample was filtered through a pre-dried and pre-weighed Whatman GF/C filter paper (Whatman, Maidstone, U.K.) in duplicate. DWs were calculated in mg/l after the filtrates were dried in an oven at 80°C overnight. Total chlorophyll (*a*+*b*) and carotenoids were found spectrophotometrically according to Lichtenthaler [21]. The absorbance value was measured at 680 nm wavelength, and cells were counted in an improved Neubauer hemocytometer by triplicate samples. Specific growth rate (µ) was calculated on both DW and cell basis according to the equation below:

$$\mu(d^{-1}) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

X_2 and X_1 in the equation represent the biomass concentrations at the times t_2 and t_1, respectively.

RESULTS

In the first trial, the biomass concentrations in both groups were similar until the 11th day, on which Na-Ac was added in the phototrophically grown culture, the TM group being slightly lower. By the addition of Na-Ac, however, the biomass started to increase faster in alternative Na-Ac application compared with the traditional Na-Ac application, and the concentration reached to 1,510 mg/l (Fig. 1). The subsequent addition of Na-Ac also supported *H. pluvialis* cells to divide, and the cell number increased from 21.7 to 42.9×10^4 cells/ml almost 2-fold during 5-day exposure to Na-Ac, whereas the increase in TM was only 1.2-fold in the same period (Fig. 2A). Similarly, the µ on DW and cell basis were much higher in the AM group between the days 11–16 compared with the previous period (Table 1). The µ

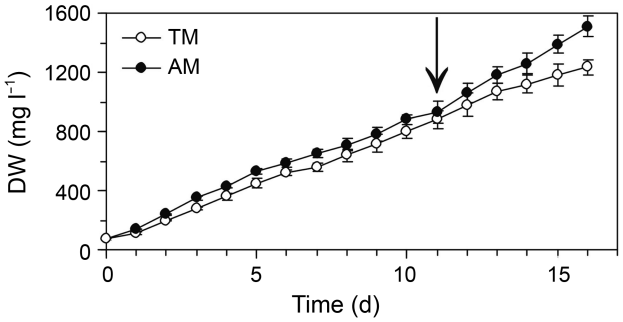

Fig. 1. Changes in DWs of TM and AM groups.
Arrow indicates Na-Ac addition in the AM group. TM, traditional mixotrophy; AM, alternative mixotrophy; DW, dry weight; Na-Ac, sodium acetate.

increased from 0.07±0.01 in TM to 0.10±0.03 d^{-1} in AM while raising from 0.04±0.02 to 0.14±0.04 d^{-1} after Na-Ac addition for DW and cell basis, respectively. The difference between the growth rates on DW and cell basis arose from the cells being triggered to divide instead of enlargement in AM.

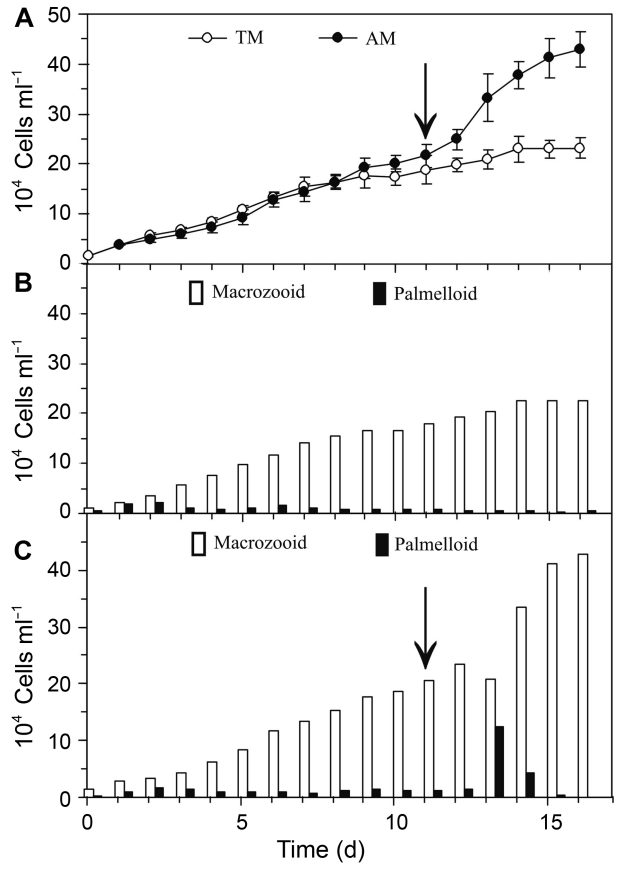

Fig. 2. Effect of Na-Ac application on cell number (**A**) and cell type in TM (**B**) and AM (**C**) groups.
Arrows (**A** and **C**) indicate Na-Ac addition in the AM group. TM, traditional mixotrophy; AM, alternative mixotrophy; Na-Ac, sodium acetate.

Table 1. Changes in growth rates (μ) of traditional mizotrophy (TM) and alternative mixotrophy (AM) groups on dry weight (DW) and cell basis.

	μ (d^{-1})			
	on DW basis		on cell basis	
Groups	Days 0–11	Days 11–16	Days 0–11	Days 11–16
TM	0.23±0.02	0.07±0.01	0.22±0.03	0.04±0.02
AM	0.24±0.02	0.10±0.03	0.24±0.04	0.14±0.04

Data are the mean±SD of three replicates.

The vegetative cells, in general, tended to grow in palmelloid form, except for the first 2 days in which maximal macrozooid cell numbers for the TM and AM groups were achieved to be 2.3×10^4 and 1.7×10^4 cells/ml respectively (Fig. 2B and 2C). However, especially on the second day of Na-Ac addition in the AM group, the macrozooid cell number sharply rose to 12.4×10^4 cells/ml and gradually dropped in the following 2 days (Fig. 2C).

Chlorophyll *a*+*b* amounts of the groups were alike until day 13 and the AM group slightly increased at the end of the experiment (Fig. 3A). The chlorophyll *a*/*b* ratio of the TM group was higher than the AM group in the whole period. In contrast to chlorophyll content, volumetric production of carotenoids in the AM group was accelerated by day 12 (Fig. 3B). The chlorophyll/carotenoids ratio in the AM group increased from 4.14 to 4.74 at the end of the experiment, whereas it was steady at 3.38±0.06 in the TM group.

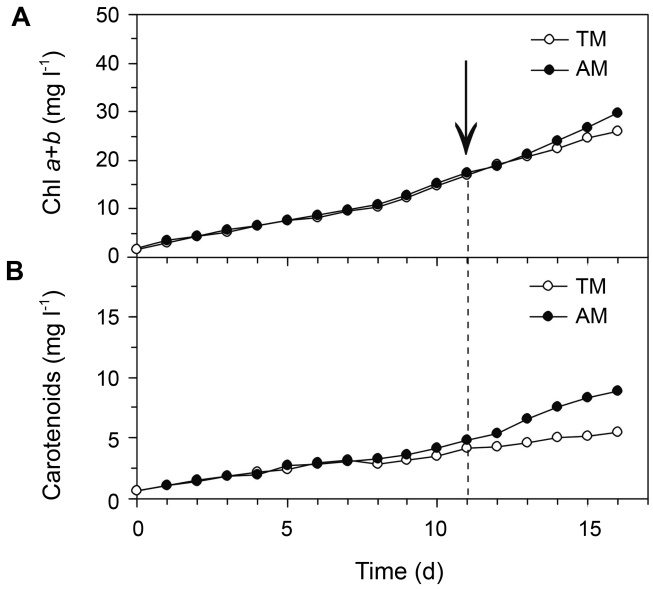

Fig. 3. Effect of Na-Ac application on chlorophyll *a*+*b* (**A**) and carotenoids (**B**) contents in TM and AM groups.
The arrow with vertical dashed line indicates Na-Ac addition in the AM group. TM, traditional mixotrophy; AM, alternative mixotrophy; Na-Ac, sodium acetate; Chl, chlorophyll.

Cell weight and chlorophyll amounts in the groups exhibited almost the same curves (Fig. 4A and 4B). Accordingly, the values in the TM group until the day 7 were lower than those in the AM group. The higher macrozooid number and the lower DW in the first days of the TM group supported this situation in this period. By the addition of Na-Ac on the 11th day in the AM group, the cell weight and chlorophyll amounts gradually decreased following two days, while keeping on increasing slightly in the TM group. In this 5-day period of AM group following Na-Ac addition, the cell diameter dropped from 27.6±0.5 to 18.6±1.2 μm in the first 2 days and reached to 26.4±0.7 μm at the end of the experiment. As for the TM group, the cell diameter showed a constant increase from 22.8±0.6 to 29.3±0.5 μm. However, carotenoids per cell were almost the same in the groups and found to be 22.2±0.3 and 20.7±0.4 pg/cell during the last 5 days for TM and AM groups, respectively (Fig. 4C).

Cell count is one of the most important parameters but also a time-consuming procedure while determining the growth. In this respect, a relationship between cell number and absorbance value at 680 nm was examined in the AM

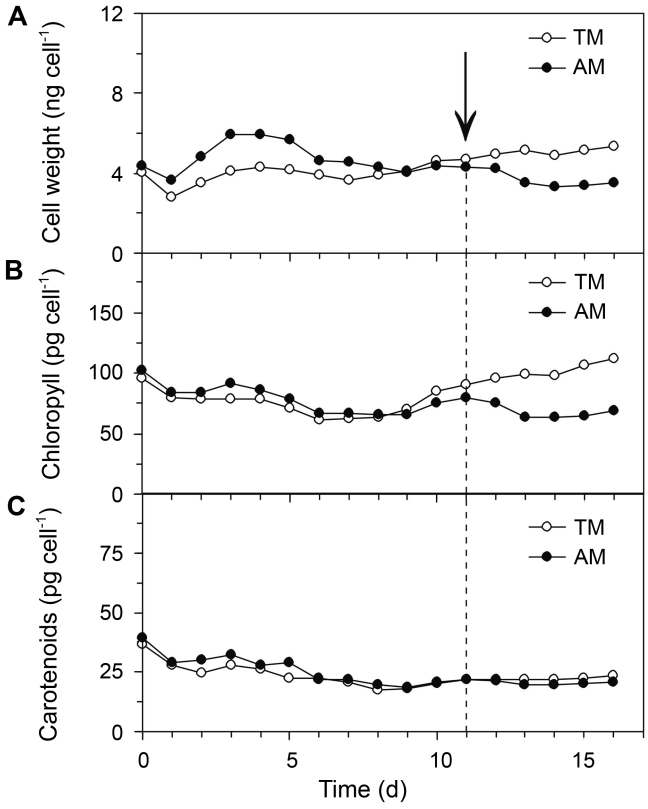

Fig. 4. Effect of Na-Ac application on the weight (**A**), chlorophyll (**B**), and carotenoids (**C**) of the cells in TM and AM groups.
The arrow with a vertical dashed line indicates Na-Ac addition in the AM group. TM, traditional mixotrophy; AM, alternative mixotrophy; Na-Ac, sodium acetate.

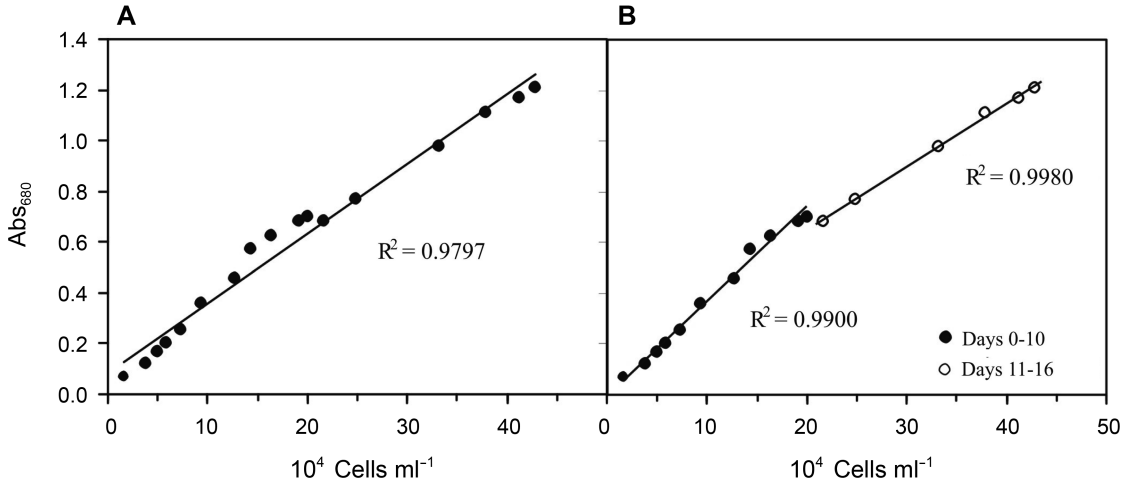

Fig. 5. Relationship between cell number and Abs $_{680}$ in the AM group.
The relationship was examined in the whole period (**A**) or in two parts (**B**) in which Na-Ac addition was the determinative factor. AM, alternative mixotrophy; Abs $_{680}$, absorbance value at 680 nm; Na-Ac, sodium acetate.

group. Although a linear correlation was found during the whole period (Fig. 5A, R^2=0.9797), the correlation became stronger when the experiment was divided into two parts (*e.g.*, before and after the Na-Ac addition) (Fig. 5B, R^2= 0.9900 and 0.9980).

In order to understand the effect of different light levels in AM, the cells were exposed to five different light regimes following Na-Ac addition (*e.g.*, dark, 20, 40, 75, and 150 µmol photons m^{-2} s^{-1}). DWs increased in parallel with increasing levels of light, and maximum cell number was achieved with 75 µmol, since 150 µmol inhibited the cells to divide especially through the first 3 days. In addition, the cells exposed to 40 µmol increased in the same ratio as 75 µmol during the first 2 days and then started to decrease after the 3rd day. As for the dark group, the cells slightly grew for the first 3 days (Fig. 6). Influences of irradiance on the other parameters such as µ, pigments, and Pv are given in Table 2.

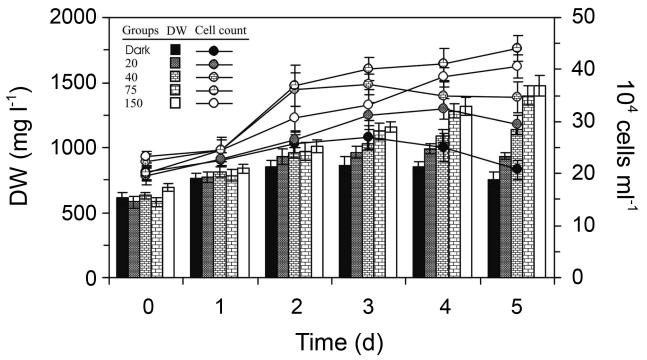

Fig. 6. Influence of irradiance on DW and cell number in AM.
AM, alternative mixotrophy; DW, dry weight.

Discussion

Haematococcus pluvialis is a microalgal species able to grow in both mixotrophic and heterotrophic regimes, so that numerous studies have been carried out with Na-Ac [3, 12, 17, 23]. Although there are contradictions on the optimum concentration of Na-Ac in mixotrophic cultures of *H. pluvialis*, most of the researchers come to an agreement that Na-Ac is growth-limiting above the optimal. Orosa *et al*. [23] reported that the addition of Na-Ac in the culture medium at 0.25% (2.5 g/l) concentration resulted in an increase of µ, above which the growth was inhibited. As for the study performed by Gong and Chen [9], the optimum Na-Ac concentration was found to be 0.51 g/l. Another study on Na-Ac nutrition of *H. pluvialis* was carried out by Cifuentes *et al*. [5] in which the concentrations varied between 4–12 mM (0.33–0.98 g/l). Accordingly, cell count slightly increased by Na-Ac addition from 1.5×10^5 cells/ml in the control group to 1.8×10^5 cells/ml in 12 mM concentration at a PFD of 85 µmol photons m^{-2} s^{-1}. In our study, the Na-Ac concentration of 1 g/l, which is considered optimal for most of the studies, was applied in the TM group, but the expected increase in cell number did not occur. In this respect, we presumed that 1 g/l was higher than the optimal for the strain 5/99 as in the study of Gong and Chen [9] who reported the optimal concentration to be 0.51 g/l.

In the study of Chen *et al*. [3], in which both mixotrophic and heterotrophic growth of *H. pluvialis* on Na-Ac was investigated, a detail called our attention. Accordingly, Na-Ac was completely consumed in mixotrophic and heterotrophic regimes in the 7th and 4th day, respectively, and the cells in the mixotrophic regime kept on growing with inorganic nutrients after Na-Ac was consumed. Similarly, Jeon *et al*.

Table 2. Influence of irradiance on growth rates (μ), pigments, and volumetric productivity (Pv) in alternative mixotrophy at the end of the trial.

	Groups				
	Dark	20	40	75	150
μ on cell basis (d^{-1})	0.21±0.02	0.23±0.01	0.24±0.01	0.26±0.02	0.25±0.01
Chlorophylls (mg/l)	12.36±1.08	23.92±0.83	25.38±1.34	22.86±1.77	22.65±1.43
Chlorophylls (pg/cell)	53.74±4.71	81.31±3.89	73.27±3.21	51.92±3.51	55.79±2.28
Carotenoids (mg/l)	4.48±0.33	6.15±0.19	6.82±0.38	6.62±0.52	7.97±0.45
Carotenoids (pg/cell)	19.48±1.36	20.90±0.77	19.69±0.87	15.04±1.02	19.63±0.81
Pv (mg l^{-1} d^{-1})	55.00±2.25	68.85±3.07	84.62±3.88	104.63±3.29	110.38±4.33

Data are the mean±SD of three replicates.

[14] reported the same phenomenon, and Na-Ac of about 1.5 g/l was consumed by *H. pluvialis* in the 3rd day of mixotrophic regime. In the frame of the studies mentioned above, it was concluded that *H. pluvialis* had a tendency to use Na-Ac with priority rather than inorganic nutrients in TM. According to our hypothesis, Na-Ac in a culture medium cannot be used efficiently in TM, and the cells in fact grow phototrophically in the last days in which Na-Ac has already been consumed and the growth is seriously limited by light owing to the mutual shading of the cells. Indeed, by the addition of Na-Ac in this stage, the cells increased from 21.7×10^4 to 42.9×10^4 cells/ml (Fig. 2A). As a result, the postponed addition of Na-Ac was shown to be a more effective way of boosting cell division even in light-limited cultures of *H. pluvialis* rather than TM.

It is clear from the experiment that Na-Ac addition to phototrophically grown cells limited by light could promote the cell division. At the end of the first trial, however, the difference in DWs between AM and TM (22%) was not proportional to that in cell number (85%), as can be seen in Fig. 1 and Fig. 2A, respectively. In our opinion, the reason why this relevancy did not occur relies on the complicated cell cycle of *H. pluvialis*. The green alga *Haematococcus pluvialis* has a plant-like cell wall consisting of glycoproteins and cellulose that is modified by the cell cycle and various conditions [30]. In the vegetative stage, the flagellates have gelatinous and transparent extracellular matrix characteristic of volvacalean motile cells [11]. The transformation of motile cells into nonmotile cells was characterized by formation of a primary wall in the extracellular matrix (at least 2-week-old flagellates). In later stages, a trilaminar sheath inside the primary wall (2 to 3-week-old aplanospores) and an amorphous secondary wall (at least 3-week-old aplanospores) are formed as well [11]. In the first trial, the cells in the TM group were expected to form primary wall and even trilaminar sheath. In the AM group, however, the new flagellates that were produced from some part of the palmelloids on the 13th day following the addition of Na-Ac lost the primary wall and trilaminar sheath. In this respect, the new cells did not have enough time to form these layers and consequently the ratio of increase in DW remained lower against cell count. The same situation was observed in chlorophyll *a*+*b* amount and the increase was similar to that of DW, slightly increasing in AM about 15% compared with TM (Fig. 3A). In our opinion, a corresponding increase in both DW and chlorophyll amounts would have taken place by the formation of new layers and chlorophyll molecules in lately produced flagellates, if the culture was sustained at least 10 more days.

In the second trial, DW and chlorophylls increased correspondingly with cell count following Na-Ac addition (Fig. 5). The cells in the second trial reached to stationary phase earlier on the 8th day because of the little difference in acclimation process. In both trials, the cells were acclimated under the same conditions (*i.e.*, during 6 days under 75 μmol photons m^{-2} s^{-1}), except for the initial DWs, which were around 250 and 70 mg/l, respectively. The difference in DWs may have caused the cells in the first trial to become light-limited, to cease logarithmic growth, and to increase in DW at the end of the acclimation period, which consequently affected the productivity of the cultures owing to the difference in physiological states. However, it is obvious from the trials that the cells were limited by light at around 22×10^4 cells/ml, and Na-Ac addition to the light-limited cultures triggered the cells to increase almost 2-fold.

In the trial, the effects of different light intensities were investigated; the cells showed a substantial increase only in the first 3 days of Na-Ac addition. Furthermore, the increase was correlated with the light intensity up to 75 μmol photons m^{-2} s^{-1}, above which the growth was inhibited. In this respect, we conclude that Na-Ac and light work synergistically, and 75 μmol photons m^{-2} s^{-1} is optimal to achieve the highest cell density. In addition, another Na-Ac application on the 3rd day may be useful to further increase the cell density, since Jeon *et al*. [14] reported that Na-Ac was consumed on the 3rd day in TM and the growth in our experiment was slowed down on the

4th day. Chlorophyll content of the cells at the end of the trial declined with increasing light (Table 2). This is normal, since dimensions of light harvesting antenna lessen and cellular chlorophyll components come to a minimum as a result of photoacclimation of microalgal cells to high light [8, 24]. On the other hand, it is known that acetate as an organic carbon source enhances both growth and astaxanthin accumulation in *H. pluvialis* [12, 23]. High light is another significant factor accelerating the accumulation of carotenoids. Although carotenoids per culture volume slightly increased with higher lights, carotenoids per cell were almost in the same level, even in the non-illuminated group. It was seen that Na-Ac made a constant contribution to the carotenoids in the cells exposed to suboptimal light levels.

In vegetative-stage cultivation, *H. pluvialis* is susceptible to contamination owing to the low growth rate and the lack of a selective media. The use of Na-Ac as an organic carbon source is a possible and effectual way to increase the growth rate. In microalgal production, however, the most significant disadvantage of using organic carbon sources is the higher probability of contamination with non-phototrophic bacteria [15, 26]. The degree of the contamination may be even more serious in large-volume cultures rather than experimental scales. In this respect, we suggest that the approach put forward in this study is a solution minimizing the risk of both algal and bacterial contamination. The cells in the AM group were exposed to Na-Ac just during a 5-day period instead of at least 10 days as in the TM group. Furthermore, the fast division of the cells in this short-term period may have a suppresser effect on the growth of bacteria, as expressed by Lee *et al*. [20].

Spectrophotometric measurement of optical density is the most usual and widespread method used to estimate biomass concentration [22]. It was seen in the experiment that the optical density measurement was a reliable method to estimate cell number, especially in the log phase of the culture when the cells were actively divided (Fig. 5A and 5B). Thus, near-infrared (NIR) spectroscopy, which has recently begun to be used in microalgae mass cultivation [27] because of its speed and accuracy, may be utilized in continuous cultivation of *H. pluvialis* for real-time monitoring of algal density.

Consequently, the late addition of Na-Ac resulted in a surprising increase of *H. pluvialis* cells (strain 5/99). The most significant advantage of the AM used in the study is to reach a much higher cell number in a batch culture period compared with the TM. In addition, the contamination risk is minimized owing to the shorter exposure time of the cells to any organic carbon source. In conclusion, this method may be applicable to the other strains of the species, and even to the other microalgae species able to grow mixotrophically.

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References

1. Aflalo, C., Y. Meshulam, A. Zarka, and S. Boussiba. 2007. On the relative efficiency of two vs. one-stage production of astaxanthin by the green alga *Haematococcus pluvialis*. *Biotechnol. Bioeng.* **98:** 300–305.
2. Boussiba, S. and A. Vonshak. 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. *Plant Cell Physiol*. **32:** 1077–1082.
3. Chen, F., H. Chen, and X. Gong. 1997. Mixotrophic and heterotrophic growth of *Haematococcus lacustris* and rheological behaviour of the cell suspensions. *Bioresour. Technol.* **62:** 19–24.
4. Christiansen, R., O. Lie, and O. J. Torrissen. 1995. Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First-feeding fry. *Aquac. Nutr.* **1:** 189–198.
5. Cifuentes, A. S., M. A. Gonzalez, S. Vargas, M. Hoeneisen, and N. Gonzalez. 2003. Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, U.S.A.) under laboratory conditions. *Biol. Res.* **36:** 343–357.
6. Fabregas, J., A. Otero, A. Maseda, and A. Dominguez. 2001. Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis*. *J. Biotechnol*. **89:** 65–71.
7. Fabregas, J., A. Dominguez, M. Regueiro, A. Maseda, and A. Otero. 2001. Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. *Appl. Microbiol. Biotechnol*. **53:** 530–535.
8. Falkowski, P. G. 1980. Light-shade adaptation and vertical mixing of marine phytoplankton, pp. 99–117. *In* P. G. Falkowski (ed.). *Primary Productivity in the Sea*. Plenum, New York.
9. Gong, X. and F. Chen. 1997. Optimization of culture medium for *Haematococcus pluvialis*. *J. Appl. Phycol*. **9:** 437–444.
10. Guerin, M., M. E. Huntley, and M. Olaizola. 2003. *Haematococcus* astaxanthin: Applications for human health and nutrition. *Trends Biotechnol.* **21:** 210–216.
11. Hagen, C., S. Siegmund, and W. Braune. 2002. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. *Eur. J. Phycol.* **37:** 217–226.
12. Hata, N., J. C. Ogbonna, Y. Hasegawa, H. Taroda, and H. Tanaka. 2001. Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic-photoautotrophic culture. *J. Appl. Phycol.* **13:** 395–402.
13. Inborr, J. 1998. *Haematococcus*, the poultry pigmentor. *Feed Mix* **6:** 31–34.
14. Jeon, Y.-C., C.-W. Cho, and Y.-S. Yun. 2006. Combined effects of light intensity and acetate concentration on the growth of unicellular microalga *Haematococcus pluvialis*. *Enzyme Microb. Tech.* **39:** 490–495.
15. Jin, E., C.-G. Lee, and J. E. W. Polle. 2006. Secondary carotenoid accumulation in *Haematococcus* (Chlorophyceae): Biosynthesis,

regulation, and biotechnology. *J. Microbiol. Biotechnol.* **16:** 821–831.
16. Kaewpintong, K., A. Shotipruk, S. Powtongsook, and P. Pavasant. 2007. Photoautotrophic high-density cultivation of vegetative cells of *Haematococcus pluvialis* in airlift bioreactor. *Bioresour. Technol.* **98:** 288–295.
17. Kobayashi, M., T. Kakizono, and S. Nagai. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. *Appl. Environ. Microbiol.* **59:** 867–873.
18. Lee, Y.-K. and C. W. Soh. 1991. Accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). *J. Phycol*. **27:** 575–577.
19. Lee, Y.-K. and D.-H. Zhang. 1999. Production of astaxanthin by *Haematococcus*, pp. 173–190. *In* Z. Cohen (ed.). *Chemicals from Microalgae*. Taylor and Francis, London.
20. Lee, H.-S., Z.-H. Kim, S.-E. Jung, J.-D. Kim, and C.-G. Lee. 2006. Specific light uptake rate can be served as a scale-up parameter in photobioreactor operations. *J. Microbiol. Biotechnol.* **16:** 1890–1896.
21. Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids: Pigments of photosynthetic membranes. *Methods Enzymol*. **148:** 349–382.
22. Meireles, L. A., J. L. Azevedo, J. P. Cunha, and F. Xavier Malcata. 2002. On-line determination of biomass in a microalgal bioreactor using a novel computerized flow injection analysis system. *Biotechnol. Prog.* **18:** 1387–1391.
23. Orosa, M., D. Franqueira, A. Cid, and J. Abalde. 2005. Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*. *Bioresour. Technol.* **96:** 373–378.
24. Prezelin, B. B. and H. A. Matlick. 1980. Time course of photoadaptation in the photosynthesis irradiance relationship of a dinoflagellate exhibiting photosynthetic periodicity. *Mar. Biol.* **58:** 85–96.
25. Rippka, R., J. B. Deruelles, M. Herdman, B. Waterbury, and R. Y. Stanier. 1979. Generic assignments, strain history and properties of pure cultures of *Cyanobacteria*. *J. Gen. Microbiol*. **111:** 1–61.
26. Rocha, J. M. S., J. E. C. Garcia, and M. H. F. Henriques. 2003. Growth aspects of the marine microalga *Nannochloropsis gaditana*. *Biomol. Eng.* **20:** 237–242.
27. Sandnes, J. M., T. Ringstad, D. Wenner, P. H. Heyerdahl, T. Kallqvist, and H. R. Gislerød. 2006. Real-time monitoring and automatic density control of large-scale microalgal cultures using near infrared (NIR) optical density sensors. *J. Biotechnol.* **122:** 209–215.
28. Torzillo, G., T. Goksan, C. Faraloni, J. Kopecky, and J. Masojídek. 2003. Interplay between photochemical activities and pigment composition in an outdoor culture of *Haematococcus pluvialis* during the shift from the green to red stage. *J. Appl. Phycol*. **15:** 127–136.
29. Torzillo, G., T. Goksan, O. Isik, and S. Gokpinar. 2005. Photon irradiance required to support optimal growth and interrelations between irradiance and pigment composition in the green alga *Haematococcus pluvialis*. *Eur. J. Phycol.* **40:** 233–240.
30. Wang, S. B., F. Chen, M. Sommerfeld, and Q. Hu. 2005. Isolation and proteomic analysis of cell wall-deficient *Haematococcus pluvialis* mutants. *Proteomics* **5:** 4839–4851.
31. Yamada, S., Y. Tanaka, M. Sameshima, and Y. Ito. 1990. Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. I. Effect of dietary astaxanthin, beta-carotene and canthaxanthin on pigmentation. *Aquaculture* **87:** 323–330.