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Effects of iron electrovalence and species on growth and astaxanthin production of *Haematococcus pluvialis**

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Abstract To increase the cell concentration and the accumulation of astaxanthin in the cultivation of *Haematococcus pluvialis*, effects of different iron electrovalencies (Fe^{2+}-EDTA and Fe^{3+}-EDTA) and species (Fe-EDTA, $Fe(OH)_x^{32x}$ and $FeC_6H_5O_7$) addition on cell growth and accumulation of astaxanthin were studied. Results show that different iron electrovalencies have various effects on cell growth and astaxanthin accumulation of *H. pluvialis*. Compared with Fe^{3+}-EDTA, Fe^{2+}-EDTA stimulate more effectively the formation of astaxanthin. The maximum astaxanthin content (30.70 mg/g biomass cell) was obtained under conditions of 18 μmol/L Fe^{2+}-EDTA, despite the lower cell density (2.3×10^5 cell/ml) in such condition. Fe^{3+}-EDTA is more effective than Fe^{2+}-EDTA in improving the cell growth. Especially, the maximal steady-state cell density, 2.9×10^5 cell/ml was obtained at 18 μmol/L Fe^{3+}-EDTA addition. On the other hand, all the various species of iron (EDTA-Fe, $Fe(OH)_x^{32x}$, $FeC_6H_5O_7$) are capable to improve the growth of the algae and astaxanthin production. Among the three iron species, $FeC_6H_5O_7$ performed the best. Under the condition of a higher concentration (36 μmol/L) of $FeC_6H_5O_7$, the cell density and astaxanthin production is 2 and 7 times higher than those of iron-limited group, respectively. The present study demonstrates that the effects of the stimulation with different iron species increased in the order of $FeC_6H_5O_7$, $Fe(OH)_x^{32x}$ and EDTA-Fe.

Keyword: astaxanthin; production; *Haematococcus pluvialis*; iron electrovalence; iron species

1 INTRODUCTION

As one of the secondary carotenoid, astaxanthin is ubiquitous in nature, especially in marine environment (Lorenz and Cysewski, 2000). It is one of the most commonly found carotenoid pigments in marine animals and widely used as a red colorant in marine aquaculture such as salmonids, shrimp, lobsters, and crayfish (Johnson and An, 1991; Meyers, 1994). Furthermore, many studies have shown that astaxanthin acts as antioxidants by quenching singlet oxygen and free radicals and much more antioxidative than β-carotene and vitamin E (e.g. Terao, 1989; Miki, 1991; Kobayashi et al., 1997). This property have arisen a wide interest in using astaxanthin to decrease the incidence of cancer and several other degenerative diseases (e.g. Mayne, 1996; Flores-Cotera and Sánchez, 2001).

Astaxanthin comes mainly from four different types of sources: artificial synthesis, abstract from shell animals, production by yeast *Phaffia rhodozma*, and production using algae (Johnson and An, 1991; Ping et al., 2007). For containing a superior amount of astaxanthin (1.5%–3.0%) in contrast and having similar composition of astaxanthin esters to that of crustaceans, green alga *H. pluvialis* is considered one of the best microbial sources of astaxanthin. *H. pluvialis* has mainly two different existing forms and the astaxanthin is accumulated in the transition between green vegetative cells and red aplanospores as the result of stress conditions (Margalith, 1999; Fábregas et al., 2001). As different culture conditions are required for the production of green vegetative cells of *H. pluvialis* and the accumulation of astaxanthin in the red aplanospores, a two-stage production process has been proposed (Harker et al., 1996; Olaizola, 2000; Fábregas et al., 2001). The conditions for the astaxanthin production are known to be considerably different from those for the growth of *H. pluvialis*: the first stage is for high-rate

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growth of green motile cells, and the second is for the hyper accumulation of astaxanthin in red cells (Harker et al., 1996; Lorenz and Cysewski, 2000; Olaizola, 2000; Choi et al., 2002).

The growth of microalgae is affected by light, temperature, nutrients (mainly N, P) and some trace elements (Maldonado and Price, 1996; Sunda and Huntsman, 1997; Naito et al., 2005; Chen and Chen, 2006). In addition to carbon, nitrogen, and phosphorous, the requirement of special trace element for specific species should be noted. Among trace elements, iron is one of the most essential elements required by microalgae because it takes part in the assimilation of nitrate and nitrite, the deoxidizing of sulphate, the fixation of nitrogen, and the syntheses of chlorophyll, and many other biological syntheses and degradation reactions (Robert et al., 1990; Zhu et al., 2000; Liu et al., 2005). Iron deficiency has been demonstrated to limit the growth of microalgae even in high nutrient environments (Naito et al., 2005). Furthermore, ferrous ion addition has been suggested promoting astaxanthin formation (Harker et al., 1996; Kobayashi et al., 1991, 1993; Choi et al., 2002).

In the present study, different iron electrovalencies (Fe^{2+}-EDTA and Fe^{3+}-EDTA) and species (Fe-EDTA, $Fe(OH)_x^{32x}$ and $FeC_6H_5O_7$), are chosen to test their effects on growth and astaxanthin accumulation of *H. pluvialis*, and to find their optimal concentrations for mass culture and optimal production of astaxanthin.

2 MATERIALS AND METHODS

2.1 Algal strain and growth

H. pluvialis was obtained from the Culture Collection of Institute of Hydrobiology, Chinese Academy of Sciences. In order to examine the effects of individual iron species and electrovalencies on the growth of *H. pluvialis*, a Bold Basal Medium (BBM medium, Nichols and Bold, 1969) was modified and used as a basal medium for maintenance cultures. The formulation of modified BBM medium is (in mmol/L): $NaNO_3$ 2.9, $MgSO_4{\cdot}7H_2O$ 0.3, NaCl 0.43, K_2HPO_4 0.43, KH_2PO_4 1.29, $CaCl_2$ 0.17, $ZnSO_4{\cdot}7H_2O$ 0.030 7, $MnCl_2{\cdot}4H_2O$ 0.007 3, MoO_3 0.004 9, $CuSO_4{\cdot}5H_2O$ 0.006 3, $Co(NO_3)_2{\cdot}6H_2O$ 0.001 7, H_3BO_3 0.18, KOH 0.55 and EDTA 0.17. All components used are analytical grade.

Erlenmeyer flasks (250 cm^3) containing 100 cm^3 of medium were used for cultivation in a photoincubator at 23±1.5°C. Continuous illumination was applied in an average light intensity of 1.5 klx under astaxanthin-noninductive conditions and 10–12 klx under astaxanthin-inductive conditions provided by cool white fluorescent lamps. Cells were inoculated at density of 6.74×10^4 cells/ml from the exponentially growing phase to additional 3 days as the precultivation. All experiments were performed in triplicate.

2.2 Culture experiment

The BBM medium was prepared as the iron-limited medium for culture experiments. The medium was freshly prepared and the pH was adjusted to 8.0 with NaOH. Different iron species of different electrovalencies were added individually. In order to get $Fe(OH)_x^{32x}$, $FeCl_3{\cdot}6H_2O$ was dissolved in distilled water (1.8–72 μmol/L). In addition, EDTA was added into ferrous iron and ferric iron solutions to get chelated iron.

As indicated in Table 1, to test the effects of different iron electrovalencies and their concentrations on cell growth and pigment synthesis, Fe^{2+}-EDTA (Group A) and Fe^{3+}-EDTA (Group B) were added separately in the culture medium at concentrations of 0, 1.8, 3.6, 18, 36 and 72 μmol/L. In subsequent experiments, to determine the effect of ascorbic acid addition on the process, four iron concentration levels, 0, 3.6, 18 and 36 μmol/L, were chosen for Fe^{2+}-EDTA+ascorbic acid (Group A1) and Fe^{3+}-EDTA+ascorbic acid (Group B1). Also, the same concentration spectrum was used to test the effect of different species of the iron on the algal growth and astaxanthin synthesis, such as $Fe(OH)_x^{32x}$ (Group C), $FeC_6H_5O_7$ (Group D) and Fe-EDTA (Group E). After the green vegetative cultures reached the stationary stage 7 days later, they were further incubated under a high light intensity (10–12 klx) for 14 days to induce the transition to the aplanospore stage and accumulation of astaxanthin. Triplicate replicates were set for each of the culture conditions.

Table 1 Experimental scheme for testing effects of various concentration of additional on the two cultures stages of *H. pluvialis* (Unit: μmol/L)

Group	Addition	0	1	2	3	4	5
A	Fe^{2+}-EDTA	0	1.8	3.6	18	36	72
B	Fe^{3+}-EDTA	0	1.8	3.6	18	36	72
A1	Fe^{2+}-EDTA+ascorbic acid	0	—	3.6	18	36	—
B1	Fe^{3+}-EDTA+ascorbic acid	0	—	3.6	18	36	—
C	$Fe(OH)_x^{32x}$	0	—	3.6	18	36	—
D	$FeC_6H_5O_7$	0	—	3.6	18	36	—
E	Fe^{3+}-EDTA	0	—	3.6	18	36	—

2.3 Analytical methods

Astaxanthin was extracted with acetone and analyzed by the method of Qi et al. (2005).

The light intensity was measured at the surface of culture tube using an illumination photometer. Cell counting was carried out daily with a haemocytometer. Growth index is represented as K value, with a formula calculation $K=(\ln N_2-\ln N_1)/(t_2-t_1)$. Algal cells were collected by centrifuging the culture fluid at 4 500 r/min for 5 min. The cell pellets were rinsed with distilled water, homogenized in agate mortar and pestle in the presence of quartz powder, extracted with acetone and centrifuged at 10 000 r/min. The supernatant was then transferred to a clean dry tube and kept in darkness. The extraction procedure was repeated at least 3–5 times until the cell debris turned almost colorless. The total pigment extracts were centrifuged again at 13 000 r/min for 15 min. All of the above processes were conducted in semi-darkness.

Astaxanthin was separated and analyzed by high-performance liquid chromatography (HPLC, Hewlett Packcard Series 1100) equipped with a DAD diode array detector using a reverse-phase 4.6×250 mm Hyper sil ODS column (Germany). The mobile phase which consists of acetonitrile/methanol/water (75:25:10, V/V/V) was used for the separation at a flow rate of 1.0 ml/min. Astaxanthin was quantified at 476 nm, and was identified using authentic standards (Sigma Co., USA).

3 RESULTS AND DISCUSSION

3.1 Effects of different iron electrovalencies on the growth and astaxanthin synthesis of *H. pluvialis*

3.1.1 Effects of different iron electrovalencies on the growth of *Haematococcus pluvialis*

The growth patterns of *H. pluvialis* in ferrous iron, ferric iron and untreated control cultures are shown in Fig.1. Compared to the iron-free group, algal growth was stimulated significantly by adding ferrous iron or ferric iron. *H. pluvialis* cultures with different Fe^{2+}-EDTA concentrations showed a content difference in growth from Day 1 of their growth curves, so did with Fe^{3+}-EDTA. As compared to that in untreated control culture (17.04×10^4 cells/ml), the cell density reached the maximum of 23.38×10^4 cells/ml in ferric iron culture and 28.63×10^4 cells/ml in ferrous iron culture on Day 5 at a proper iron concentration of 18 μmol/L, and the maximum growth rate in logarithmic phase was also gained which is much higher than that of other groups.

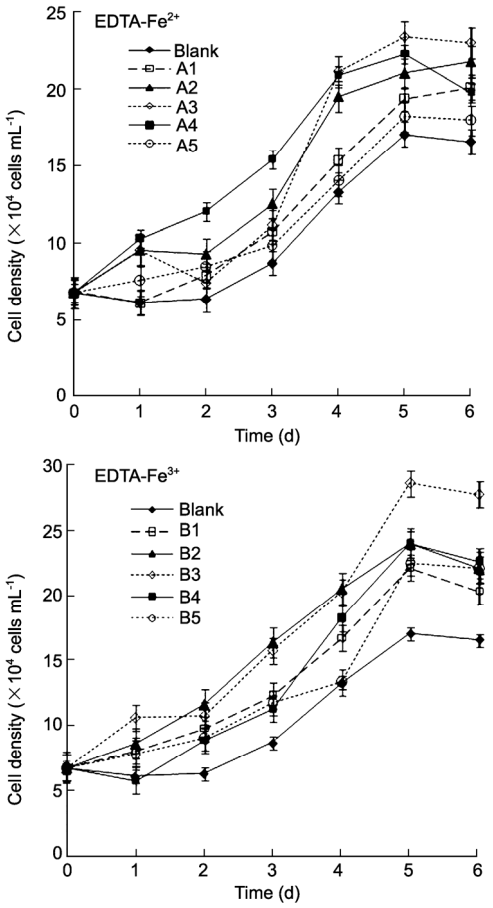

Fig.1 Comparison of the growth of *H. pluvialis* with different iron concentrations under different electrovalencies

Jin et al. (1996) reported a suitable concentration of ferrous iron for *H. pluvialis* at 0.5 mg/L, however, that of this study is 5 mg/L. The difference may be resulted from the medium and the algal strain. As it is known, ferric iron cannot be absorbed by the alga unless it was oxidized to an available form. A close relationship was revealed between the reductase in the algal cell and the adsorption of ferric iron (Ou et al., 2002). Fe^{3+}-EDTA was more effective on stimulating the *H. pluvialis* growth than Fe^{2+}-EDTA, as the culture medium we took was subacid, resulting in a more available reduction environment for ferric iron to be reduced to ferrous iron by oxidation reduction potential. Once the reduction rate of the ferric iron went beyond the adsorption rate of the ferrous iron, the ferric iron would turn to be superior over ferrous iron in promoting the algal growth.

Also, since the transformation of ferric iron is able to improve the growth of the alga, the effect of ascorbic acid on the process was examined (Table 2). However as it is indicated, no obvious improvement was observed, which is far from the Ou's (2002). It might be due to the subacid culture medium (BBM)

in which ferric iron is transformed and absorbed smoothly, and so adding the ascorbic acid is not necessary.

3.1.2 Effects of different iron electrovalencies on astaxanthin synthesis of *H. pluvialis*

The astaxanthin profile of different cultures grown under stress conditions, and the effect of different iron electrovalencies on production of astaxanthin of *H. pluvialis* are shown in Fig.2. As it indicated, the production of astaxanthin (per unit volume) in Group B with ferric iron persisting was 12.61 mg/L in average, higher than that in Group A with the addition of ferrous iron (10.56 mg/L in average). In contrast, on cellular or weight basis, the astaxanthin content in Group A (ferrous iron medium) was higher than that in Group B (ferric iron medium), which might be due to the stimulation of ferrous iron on astaxanthin synthesis in cells of *H. pluvialis*. Furthermore, there was no obvious stimulation neither in growth nor in astaxanthin formation when the concentration of ferrous iron was low (1.8 μmol/L), while a high concentration of ferrous iron (72 μmol/L) resulted in slight inhibition on the cell growth. Content of astaxanthin produced per unit weight reached a level of 25.65 mg/g, which was even more than others, through the maximal cell density was only 0.25×10^4 cell/ml. As known to us, the free radical was formed by an iron-carotene in *Dunaliella* (Ben-Amotz and Avron, 1983) and astaxanthin in the yeast *Prhodozyma* (Harker et al., 1996). Kobayashi (1993) reported that increased astaxanthin formation caused by ferrous iron was inhibited by NaI which scavenges HO·, suggesting the HO· formed by an iron catalyzing. On the other hand, Fenton reaction was required for enhancing astaxanthin biosynthesis in *H. pluvialis*. Since ferric iron might not play a role in Fenton reaction, it could not affect the astaxanthin accumulation (Borowitzka et al., 1991; Harker et al., 1996).

3.2 Effects of different iron species on the growth and astaxanthin synthesis of *H. pluvialis*

3.2.1 Effects of different iron species on the growth of *H. pluvialis*

Three sources of iron species, were supplied in the forms of Fe^{3+}-EDTA, $Fe(OH)_x^{32x}$, and $FeC_6H_5O_7$, respectively, to tested the effects of different iron species on the algal growth (Fig.3).

It was observed that the algal growth rate increased more or less according to all these types of iron, although the effects differed in different species. Compared to the blank group with iron-limited culture, all the test groups had an exponential phase, lasting from 4 to 7 days, and the maximal cell density could be obtained at the Day 5 or Day 6.

Table 2 Effect of different electrovalencies on K value of *H. pluvialis* (Unit: μmol/L)

Group	Concentration			
	0	3.6	18	36
EDTA-Fe^{2+}	0.15	0.19	0.20	0.18
EDTA-Fe^{3+}	0.15	0.21	0.22	0.21
EDTA-Fe^{2+}+ascorbic acid	0.15	0.19	0.20	0.24
EDTA-Fe^{3+}+ascorbic acid	0.15	0.21	0.21	0.16

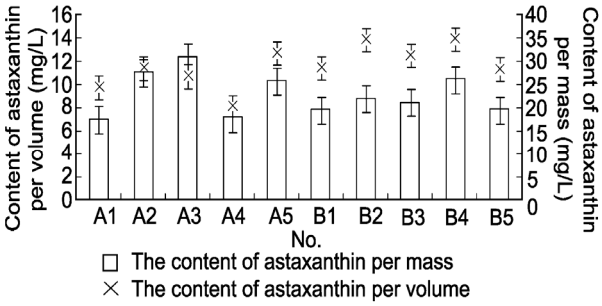

Fig.2 Effect of different iron electrovalencies on content of astaxanthin of *H. pluvialis*

$Fe(OH)_x^{32x}$, generally had a bit improvement on the algal growth, and the effect varied with the concentration of $Fe(OH)_x^{32x}$ (Fig. 1). Some previous studies have demonstrated that $Fe(OH)_x^{32x}$ could accelerate the growth rate of microbe. However, $Fe(OH)_x^{32x}$ presents as the form of $Fe(OH)_x^{32x}$, when presenting purely, which has a low solubility and should be converted to another available from so that it could be taken up by the alga. In fact, bioavailable dissolved $Fe(OH)_x^{32x}$ concentrations in medium are so low that they could not reach the level supportive of the growth of alga (C:N:P:Fe=106:16:1:0.58). Due to its adsorption characteristic, even a high level of $Fe(OH)_x^{32x}$ might restrain the growth of alga (Liu et al., 2005). Moreover, pH value played an important role in influencing the utilization of $Fe(OH)_x^{32x}$. In summary, words above are the reasons why $Fe(OH)_x^{32x}$ could not improve greatly the growth of *H. pluvialis*, which was the same with the result of Ou's (2002) focusing on the growth of *Chlorella.*

The present study shows that the most obvious improvement was observed in the test of $FeC_6H_5O_7$. The conclusion about the influence of different species iron on the growth of diatom reported by Li and Wang (1998) is not the same as ours, because they tested only on the low concentration (0.5 mg/L)

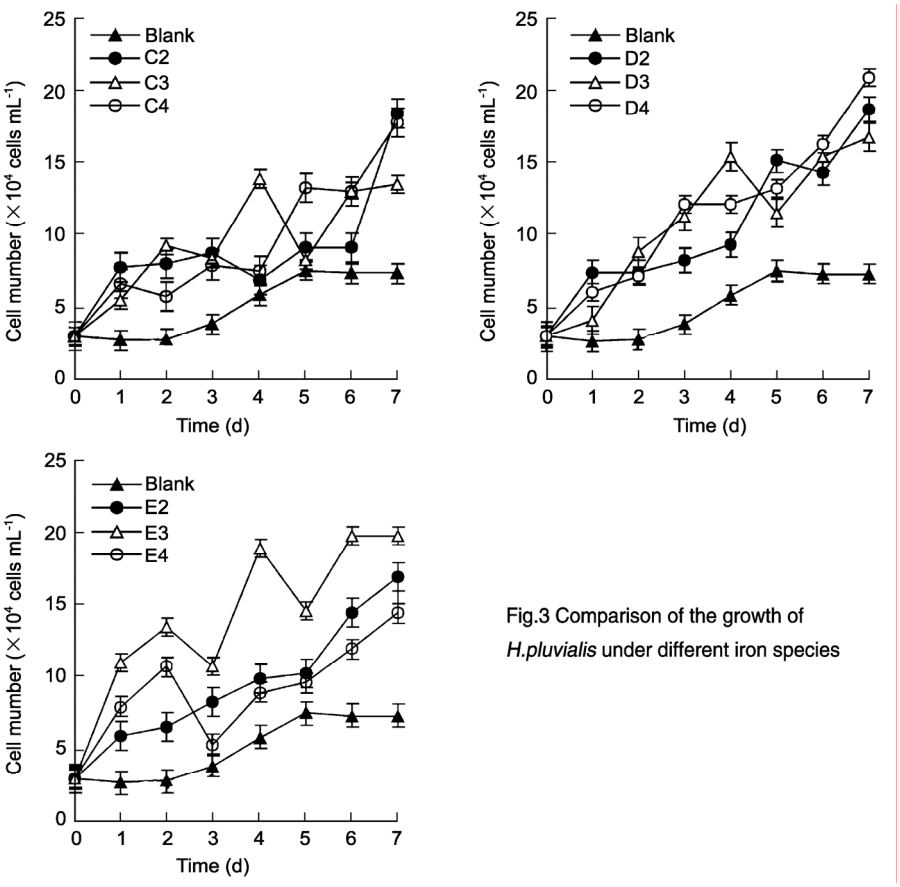

Fig.3 Comparison of the growth of *H.pluvialis* under different iron species

Table 3 Effect of different species of iron on *K* value of *H. pluvialis* (Unit: μmol/L)

Group	Concentration			
	0	3.6	18	36
$Fe(OH)_x^{32x}$	0.15	0.26	0.21	0.25
$FeC_6H_5O_7$	0.15	0.26	0.25	0.27
Fe^{3+}-EDTA	0.15	0.25	0.27	0.23

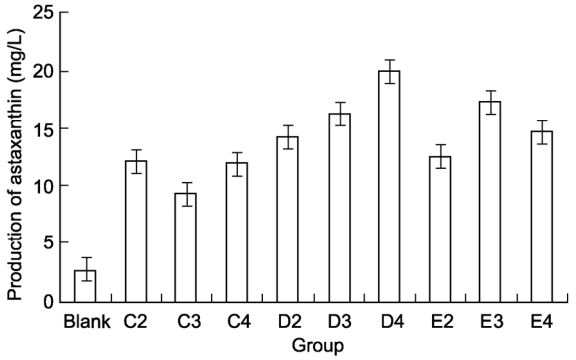

Fig.4 Effects of different iron species on the livability and aplanospores ratio of *H. pluvialis*

of the iron. Our results indicate that the convenient concentration of the iron is largely different between $FeC_6H_5O_7$ (36 μmol/L) and Fe^{3+}-EDTA (18 μmol/L), the same to the conclusion in Wang and Wang (2002). Both Li and Wang (1998) and Wang and Wang (2002) found that the convenient concentration of $FeC_6H_5O_7$ is higher than that of Fe^{2+}-EDTA and Fe^{3+}-EDTA.

3.2.2 Effects of different iron species on the astaxanthin synthesis of *H. pluvialis*

Compared to blank group, all of the tested species enhanced the astaxanthin production (Fig.4). Among the three iron species, $FeC_6H_5O_7$ is the most effective with the level of astaxanthin ranging from 14.28 mg/L to 19.98 mg/L (16.84 mg/L in average), followed by Fe^{3+}-EDTA (12.54–17.33 mg/L, 14.85 mg/L in average), and then $Fe(OH)_x^{32x}$ (9.31–12.61 mg/L, 11.16 mg/L in average). This sequence almost accords with that in culture stage where the alga had accumulated a certain quantity of cells, and the astaxanthin production was based on the quantity. Therefore, the result that the factors tested cannot stimulate the synthesis of astaxanthin might due to the level of astaxanthin accumulation depending on the biomass acquired at the end of the growth stage.

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