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Enhancing the growth rate and astaxanthin yield of *Haematococcus pluvialis* by nuclear irradiation and high concentration of carbon dioxide stress

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Abstract:

Unicellular green microalgae *Haematococcus pluvialis* was mutated with ^{60}Co–γ irradiation to promote growth rate and increase astaxanthin yield under high concentration of CO_2 stress. The average specific growth rate of *Haematococcus pluvialis* mutated with 4000Gy γ-ray irradiation was increased by 15% compared with the original strain with air aeration. The mutant grew best with 6% CO_2 (the maximum specific growth rate was 0.60/d) when it was cultured with high concentrations of CO_2 (2-10%). The peak biomass productivity (0.16g/L/d) of the mutant cultured with 6% CO_2 was 82% higher than that of the mutant with air. The astaxanthin yield and lipid content of the mutant induced with 6% CO_2 and high light (108 μmol photons m^{-2} s^{-1}) increased to 46.0mg/L and 45.9%, which were 2.4 and 1.3 times higher than those of the wild-type strain, respectively.

Keyword: *Haematococcus pluvialis*; Nuclear mutagenesis; High CO_2; Astaxanthin; Growth rate

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1. Introduction

Astaxanthin (3, 3′-dihydroxy-diketo-β, β′- carotene-4, 4′-dione) is one of the most powerful antioxidants among carotenoids with many applications in nutraceuticals and in the food and feed industries because of its strong anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on organisms (Lee et al., 2015). The unicellular green microalgae *Haematococcus pluvialis*, with an astaxanthin content of 1–5%, is recognized as the best biological source of natural astaxanthin, and this species is the only commercialized microalgae in astaxanthin production. However, the specific growth rate of *Haematococcus pluvialis* is only 0.15/d-0.64/d (Orosa et al., 2005; Kaewpintong et al., 2007; Imamoglu et al., 2010), and this low growth rate causes biological contamination, which hinders large-scale cultivation.

Genetic transformation and mutation with physical or chemical mutagens have been used to obtain astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. The specific growth rate and astaxanthin content of mutant strains obtained by ultraviolet (UV) exposure and by ethyl methane sulphonate, *1*-methyl-*3*-nitro *1*-nitrosoguanidine or *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatments can reach 0.031/h-0.08/h and up to 5.8% of cell dry weight, respectively (Kamath et al., 2008; Hu et al.,2008; Peng et al., 2008; Wu et al.,2010; Li et al., 2010; Hong et al., 2012; Gomez et al., 2013). Astaxanthin content can be increased by two-or threefold through cloning and genetic transformation of β-carotene ketolase genes(*bkt*) and phytoene desaturase genes(*pds*) in *Haematococcus pluvialis* (Steinbrenner et al., 2006;

Wang et al., 2012; Kathiresan et al., 2015; Sharon-Gojman et al., 2015). γ-Ray has been recently used to promote the growth rate and metabolite yield of microalgae for its strong penetration considerably surpassing that of UV and X-ray. γ-Rays and free radicals generated via radiolysis of water can improve the photosynthesis of microalgae cells by activating RuBisCO enzymes, and enhancing C3 production in the Calvin–cycle. Previous studies found that ^{60}Co-γ irradiation increased the biomass yield of *Chlorella pyrenoidosa* by 53.1% and the starch accumulation of *Spirogyra varians* by threefold (Cheng et al., 2013; Yoon et al., 2013). Astaxanthin-hyperproducing strains of *Phaffia rhodozyma* can be obtained through chromosomal rearrangement caused by ^{60}Co-γ irradiation and screening by oxygen radicals (Sun et al., 2004; Najafi et al., 2008). Thus, ^{60}Co-γ irradiation may simultaneously increase biomass and astaxanthin productivity. However, improving *Haematococcus pluvialis* strains via ^{60}Co-γ irradiation has not been attempted.

High concentrations of CO_2（≥5%）have been used as a carbon source culturing *Haematococcus pluvialis* in many studies for its advantages in restraining biological contamination and promoting photosynthesis (Kang et al., 2010; Lee et al., 2015; Giannelli et al., 2015). High CO_2（≥5%）can also be an environmental stress inducing astaxanthin accumulation at the red stage. A previous study measured an astaxanthin concentration of 29.62 mg/g in distilled water with CO_2, with no significant difference with that in N-free medium (Imamoglu et al., 2009). Kang et al. (2005) reported that the astaxanthin yield of *Haematococcus pluvialis* induced with 5% CO_2 (77.2 mg/g) was 3.4-fold higher than that with heterotrophic induction. Nevertheless, the growth

rate of *Haematococcus pluvialis* with 5% CO_2 (0.232/d) remains to be improved. Yin et al. (2015) reported that CO_2 concentration above 1.5% suppressed biomass and astaxanthin yield. Thus, further studies on growth rate stimulation of *Haematococcus pluvialis* under high CO_2 and on the response of cells to high CO_2 are necessary.

In the present study, a fast-growing *Haematococcus pluvialis* mutant was obtained via ^{60}Co-γ irradiation, and the growth rate of the mutant was further increased with high concentration of CO_2. The astaxanthin and lipid yield were enhanced by the synergic induction of high concentration of CO_2 and high light. The ultrastructure of the *Haematococcus pluvialis* mutant cells at the green logarithmic phase and at the photoautotrophic induction period with high CO_2 were observed via transmission electron microscope (TEM).

2 Materials and Methods

2.1 *Haematococcus pluvialis* strain and growth medium

The microalgae strain used in this study was *Haematococcus pluvialis* FACHB-872 purchased from the Freshwater Algae Culture Collection of Hydrobiology, Chinese Academy of Sciences, China. Microalgae cells were cultured in Bold's Basal Medium (BBM) containing $2.94 \cdot 10^{-3}$ M $NaNO_3$, $3.04 \cdot 10^{-4}$ M $MgSO_4 \cdot 7H_2O$, $4.28 \cdot 10^{-4}$ M NaCl, $4.31 \cdot 10^{-4}$ M K_2HPO_4, $1.29 \cdot 10^{-3}$ M KH_2PO_4, $1.70 \cdot 10^{-4}$ M $CaCl_2 \cdot 2H_2O$, $3.07 \cdot 10^{-5}$ M $ZnSO_4 \cdot 7H_2O$, $7.28 \cdot 10^{-6}$ M $MnCl_2 \cdot 4H_2O$, $4.93 \cdot 10^{-6}$ M MoO_3, $6.29 \cdot 10^{-6}$ M $CuSO_4 \cdot 5H_2O$, $1.68 \cdot 10^{-6}$ M $Co(NO_3)_2 \cdot 6H_2O$, $1.85 \cdot 10^{-4}$ M H_3BO_3, $1.71 \cdot 10^{-4}$ M EDTA $\cdot Na_2$, $5.53 \cdot 10^{-4}$ M KOH and $1.79 \cdot 10^{-5}$ M

$FeSO_4 \cdot 7H_2O$ (Dominguez-Bocanegra et al, 2004). Cultures were incubated at 25℃ and 33 μmol photons m^{-2} s^{-1} of continuous light and were shaken manually twice a day.

2.2 Mutagenesis and isolation of mutants

Motile cells at the logarithmic growth phase of *Haematococcus pluvialis* were irradiated at various doses (250、500、1000、2000、3000、4000、5000Gy) using a ^{60}Co-γ irradiator in Zhejiang Academy of Agricultural Science and then kept for 12 h in the dark. The mutants were cultured in an incubator at 22 ℃ and 18 μmol photons m^{-2} s^{-1} of light under a 12 h: 12 h light/dark cycle for recovery. After the γ-irradiated strains revived, cells were plated on BBM agar plates and then cultured at 25℃ and 33 μmol photons m^{-2} s^{-1} of continuous light. Large colonies with dark-red color were selected for further culturing in flasks and the strain with the highest growth rate was picked out for further studies.

2.3 Growth and astaxanthin accumulation of the mutant under high CO_2

Mutants at the logarithmic growth phase were inoculated into 400 mL fresh BBM medium in glass cylinder photobioreactors (diameter: 8.2 cm, height: 15.3 cm) covered with silica gel stoppers. Cultures were aerated continuously with 0.04% (air), 2%, 6%, 8% and 10% CO_2 (v/v) at a rate of 120mL/min (0.3vvm). Cultures were incubated at 25℃ and 45 μmol photons m^{-2} $s^{-1}$of continuous light, and the growth curves at the green stage were measured. Afterward, cells at the stationary phase were induced under continuous CO_2-supplied conditions at 2%, 6%, 8% or 10%

concentration with low light illumination (45 μmol photons m^{-2} s^{-1}) for 10 days. Astaxanthin yields were measured every 2 days, and cells were sampled every 5 days for TEM. Cultures were subjected to a high light intensity (108 μmol photons m^{-2} s^{-1}) for 15 days, and the final astaxanthin yield and lipid content were measured. All measurements were repeated in triplicate and a mean value was reported.

2.4 Analytical methods

2.4.1 Growth analyses

Cell growth was monitored by determining the absorbance of samples at 680 nm (OD680) in a UV/Visible spectrophotometer (Unico, 2600UV/VIS, USA). For final dry weight determination, 10ml cell cultures were dewatered by centrifugation (BeckmanAvanti J26-XP, USA) and the pellets were washed with distilled water twice, then microalgae pellets were collected and dried at 80°C for 24 h. The biomass dry weight (BDW) at the logarithmic growth phase was calculated as: BDW (g/L) = 0.512 $\times OD_{680}$. Biomass productivity (P) was calculated as: $P = (M_1 - M_0) / (t_1 - t_0)$. Specific growth rate was calculated as: $\mu = (\ln M_1 - \ln M_0) / (t_1 - t_0)$, where M_1 was the dry weight at time t_1 and M_0 was the dry weight at time t_0.

2.4.2. Astaxanthin determination

Astaxanthin yield was measured by spectrophotometric method (Boussiba et al., 1992). Cell pellets harvested via centrifugation were resuspended in a solution of 5% (w/v) KOH in 30% (v/v) methanol and then heated in 70°C water bath for 10 min to destroy chlorophyll. The mixture was centrifuged and the remaining pellet was

extracted with a mixture of DMSO and acetic acid. After heating in 70°C water bath for 10 min, the mixture was centrifuged and the supernatant was collected. The extraction procedure was repeated until the cell debris was almost colorless. The absorbance of the combined extracts was determined at 490 nm, and the per unit volume astaxanthin concentration (c) was calculated as:

c (mg/L) = 4.5 $\times A_{490} \times V_a / V_b$

where V_a (L) was the volume of extracts, V_b (L) is the volume of the culture sample, and A_{490} is the absorbance of the extracts at 490 nm.

2.4.3 Lipid extraction and estimation

Lipids were extracted through direct transesterification (Johnson and Wen，2009). Algal biomass dried in a baking oven at 80°C for 24 h. After grinding, the dried algal biomass (0.1 g) was mixed with chloroform (4 mL), methanol (4 mL) and sulfuric acid (0.2 mL) in a digestion reactor. The digestion reactor was sealed and heated in the baking oven at 90°C for 6 h. After the reaction was completed, the mixture was allowed to cool to room temperature. The product was mixed with distilled water in a centrifuge tube and then centrifuged. After the chloroform was evaporated in a baking oven at 60°C, the dried lipids were measured gravimetrically.

2.4.4 Pigment extraction and determination

Pigment content was determined using a spectrophotometric method (Pruvost et al., 2011). Cell pellets harvested via centrifugation were resuspended in methanol (99.9%) for 30 min in the dark at 45°C. Samples were centrifuged before measurements and the absorbance of the supernatant was determined at 480 nm,

652nm and 665nm. Chlorophyll-a (Chl-a), chlorophyll-b (Chl-b) and carotenoid concentrations were determined according to the equations as follows:

[Chl-a] mg/L = $16.5169 \times A_{665} - 8.0962 \times A_{652}$

[Chl-b] mg/L = $27.4405 \times A_{652} - 12.1688 \times A_{665}$

[Carotenoids] mg/L = $4 \times A_{480}$

2.4.5 Ultrastructural analysis of mutant cells

Motile cells and red aplanospores were observed with a transmission electron microscope (TEM, 782, GTONTORN, USA). The images were analyzed using NIS-Elements software (BR 4.10.00, Nikon, Japan).

2.4.6 Measurement of CO_2 fixation rate

The **CO_2** fixation rate (R_{CO2}) was calculated from the carbon content of the biomass and the specific growth rate (μ) as follows:

$R_{CO2} = \mu \times C_C \times \text{dry weight} \times M_{CO2}/M_C$

Where C_C, M_{CO2} and M_C are the carbon content of the biomass (%), molecular weights of CO_2 and elemental carbon, respectively (Aishvarya et al., 2012).

3 Results and discussion

3.1. Growth of *Haematococcus pluvialis* mutated by ^{60}Co-γ irradiation

The wild *Haematococcus pluvialis* strain was treated with a wide dose range of ^{60}Co-γ ray (250-5000Gy). Low doses of irradiation (250-1000Gy) gave a high survival rate but a low probability of hyper-producing mutants. By contrast, a high dose of irradiation (5000Gy) caused irreversible damage to cells and hindered growth.

The mutant isolated after exposure to 4000Gy irradiation yielded highest biomass which was at least 50% higher than those of strains exposed to any other doses of irradiation, and this strain was taken for further studies. The growth rates of the mutant and the wild type with air aeration are shown in Fig.1. Compared with those of the wild type, the specific growth rate of mutant increased by 15% and the average biomass productivity increased from 0.07g/L/d to 0.09g/L/d at the logarithmic growth phase. γ-Rays can penetrate cells and enhance the expression of photosynthetic enzymes such as RuBisCO, which is a key enzyme for carbohydrate synthesis, and glutamate-1-semialdehyde-2,1-aminomutase, which is involved in 5-aminolevulinic acid synthesis. The expression of light-harvesting chlorophyll a/b binding protein was also up-regulated. Hence, the Calvin–Benson cycle was promoted, and the photosynthetic rate was accelerated. Energy metabolism-related proteins may also be up-regulated .e.g. potential growth of γ-ray mutagenesis strains could be higher than the wild type resulting from the increasing ATP synthase levels (Cheng et al., 2013; Yoon et al., 2013).

3.2. Tolerance of the *Haematococcus pluvialis* mutant to high CO_2

3.2.1 Growth of the mutant with various concentrations of CO_2

The biomass dry weight, productivity and culture pH values of the mutant with 0.04%, 2%, 6%, 8% and 10% CO_2 are shown in Fig.2. The tolerance of *Haematococcus pluvialis* to high concentration of CO_2 increased after nuclear irradiation. The highest biomass dry weight (0.65g/L) and biomass productivity (0.16g/L/d) were obtained with 6% CO_2, which were 30% and 82% higher than those

in air condition. The average and maximum specific growth rates with 6% CO_2 were 0.38/d and 0.60/d, respectively. However, the growth rate of the mutant declined under the conditions of 8% and 10% CO_2 aeration. At moderate CO_2 concentrations (2% and 6%), the pH value was maintained approximately 7, which was suitable for the growth of *Haematococcus pluvialis*. By contrast, the growth of the microalgae was hindered under a low CO_2 concentration resulting in a high pH (approximately10) and under a high CO_2 concentration (10%) resulting in a low pH (below 6.6).

The chlorophyll and carotenoid contents of the mutant with 6% CO_2 were 21.1 mg/L and 7.2 mg/L, respectively, which were 23% and 63% higher than those of the wild type with air (Fig.3). The peak chlorophyll content of mutant was obtained on the fourth day and thus photosynthesis was enhanced dramatically. The peak CO_2 fixation rate (0.61 g/L/d) of the mutant with 6% CO_2 was 2.1 times higher than that of the wild type with air.

3.2.2 Response of mutant ultrastructures to elevated CO_2 level

The vacuoles and starch granules in *Haematococcus pluvialis* mutant cells cultured with various CO_2 concentrations (0.04-10%) were observed with TEM (Fig.4). The proportion of vacuoles gradually increased from 14.1% to 33.5% when the CO_2 concentration was increased from 0.04% to 10%. Vacuoles are the main sites storing H^+ in plant cells and play an important role in controlling intracellular pH which is essential for a high CO_2 tolerance. The increase of vacuoles might result from the increasing protons which were pumped from the cytoplasm into the vacuoles via V-ATPase associated with the tonoplast (Solovchenko and Khozin-Goldberg,

2013). The highest starch granule proportion obtained was with 6% CO_2. The starch granule proportion increased from 2.3% to 12.0% with the elevating CO_2 level from 0.04% to 6%. However, the starch granule proportion declined to 4.8% when the CO_2 concentration was further increased to 10%. These findings suggested that starch accumulation was influenced by both CO_2 concentration and microalgae tolerance to elevated CO_2 levels. At relatively low CO_2 concentrations (0.04%-6%), the photosynthesis rates were enhanced by the up-regulated expression of RuBisCO promoted by the sufficient inorganic carbon. However, the increase in CO_2 concentration also decreased medium pH value. In particular, the pH value was approximately 6.5 with 10% CO_2, which was lower than that with 6% CO_2 (approximately 7). For high CO_2 adaptation, cells temporarily reduced the synthesis of organic carbon and provided more ATP to maintain the intracellular pH stability through gene regulation and increasing the energy allocation proportion PSI/PSII (Zhao et al., 2014).

3.3. Astaxanthin and lipid accumulation of the mutant with high CO_2

Haematococcus pluvialis mutant cells were induced with high concentration of CO_2 (2-10%) under low light illumination (45μmol photons m^{-2} s^{-1}) for 10 days, and the ultrastructural changes of mutant cells were observed via TEM. The proportions of starch granules were considerably higher at the red stage than that at the green stage, but they decreased by 23-51% during the induction period, whereas, the proportion of oil droplets increased two- to fourfold compared with the initial cells (Fig.5). These results suggested carbon reallocation from starch-based to lipid-based

carbon storage. *Haematococcus pluvialis* under photoautotrophic induction with high CO_2 was intensively producing starch at first and then sharply degrading starch to support lipid synthesis (Recht et al., 2012). In addition, the productivity of starch and lipid, and their metabolic trade-off might differ depending on CO_2 concentration. Starch granules increased with the increasing CO_2 concentration from 2% to 8%, and then decreased from 23.8% (8% CO_2) to 21.0% (10% CO_2) because of the toxic effect of 10% CO_2 on photosynthesis. The highest proportion of oil droplets obtained was with 6% CO_2, however, this proportion declined from 42.0% to 36.2% when the CO_2 concentration was increased from 6% to 10%. This finding might be attributed to the fact that a low pH results in few available bicarbonates for the carboxylation of lipid synthesis (Chiu et al., 2009). The interaction between astaxanthin and lipid biosynthesis pathway was feedback- coordinate at the metabolite level. Diacylglycerol acyltransferase, a key enzyme for triacylglycerol synthesis, might be the candidate enzyme catalyzing astaxanthin esterification, which drives the formation and accumulation of astaxanthin (Chen et al., 2015). In addition, astaxanthin is mainly deposited in globules made of triacylglycerol. Therefore, astaxanthin accumulation was strongly affected by lipid production. The highest astaxanthin yield was obtained with 6% CO_2 (21.6mg/L) which was 28% higher than that with 2% CO_2 and 38% higher than that with 10% CO_2 (Fig.6a).

The end-point astaxanthin yield and lipid content after 15 days induced with high concentration of CO_2 and high light were tested (Fig. 6b). The astaxanthin yield under this condition was twice to triple that under low light. The highest astaxanthin yield

was obtained with 6% CO_2 (46.0mg/l) and was about 30% higher than that obtained with 2% and 10% CO_2. No significant difference was observed between the highest (45.9%, 6% CO_2) and the lowest lipid content (43.7%, 10% CO_2) suggesting that the influence of CO_2 concentration on lipid content was limited under high light. The astaxanthin yield of the wild type was also promoted by high CO_2 concentration. The astaxanthin yield (19.5mg/L) and lipid content (36.1%) of the wild type simultaneously induced by high light and 6% CO_2 were remarkably higher than those induced by high light alone. However, the astaxanthin yield and lipid content of the wild type were only 43% and 79% those of the mutant with 6% CO_2. The biomass dry weight (2.36 g/L) of mutant induced with 6% CO_2 and high light of 108 μmol photons $m^{-2}s^{-1}$ was much higher than that (1.82g/L) with low light of 45 μmol photons m^{-2} s^{-1}. The mutant with higher growth rate yielded much more biomass (2.36 g/L) with 6% CO_2 and high light of 108 μmol photons $m^{-2}s^{-1}$ than the wild type (1.53 g/L). This was because the red cells had higher photosynthetic capacities than green cells, and astaxanthin protected D1 protein of PSII from decline with high light. Once D1 protein increased to normal level, the growth of red cells resumed and astaxanthin accumulation stopped in the cells (Wang et al, 2003).

4. Conclusions

Nuclear mutagenesis with ^{60}Co–γ irradiation combined with high concentration of CO_2 stress effectively enhanced biomass and astaxanthin yield of *Haematococcus pluvialis*. The growth rate of mutant was 15% higher than that of the original strain. The biomass productivity of mutant with 6% CO_2 was 82% higher than that of mutant

with air. The astaxanthin yield and lipid content of mutant induced with 6% CO_2 and high light were 2.4 and 1.3 times higher than those of the wild-type strain, respectively. It is necessary to study on metabolic pathways of CO_2 fixation and astaxanthin accumulation in the mutant cells.

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List of Figures and Tables:

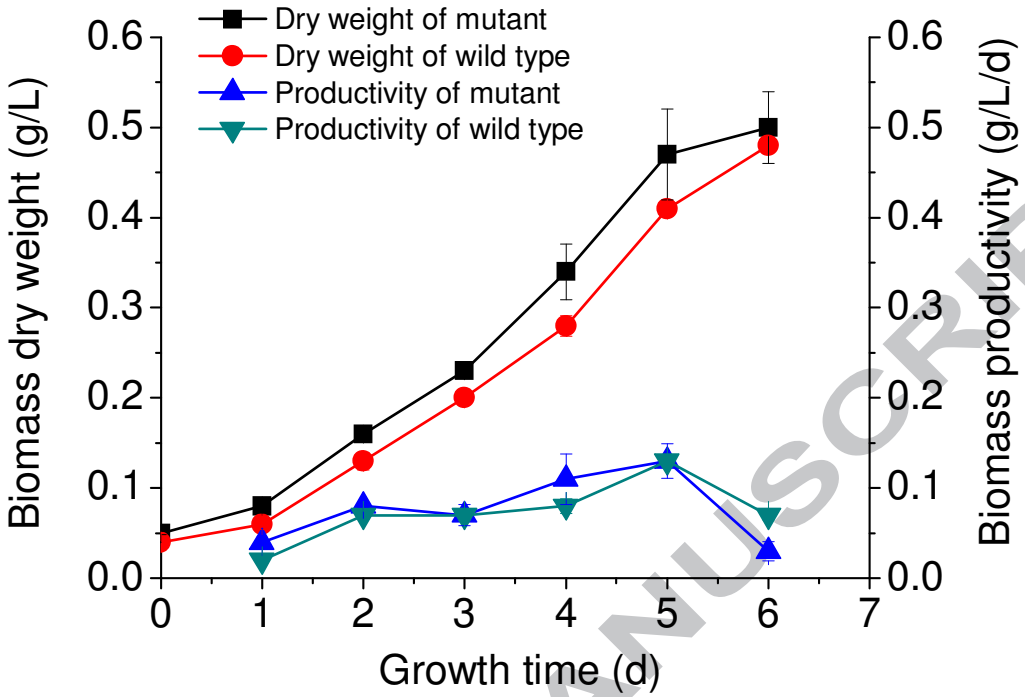

Fig.1. Growth curves of *Haematococcus pluvialis* mutant with air aeration

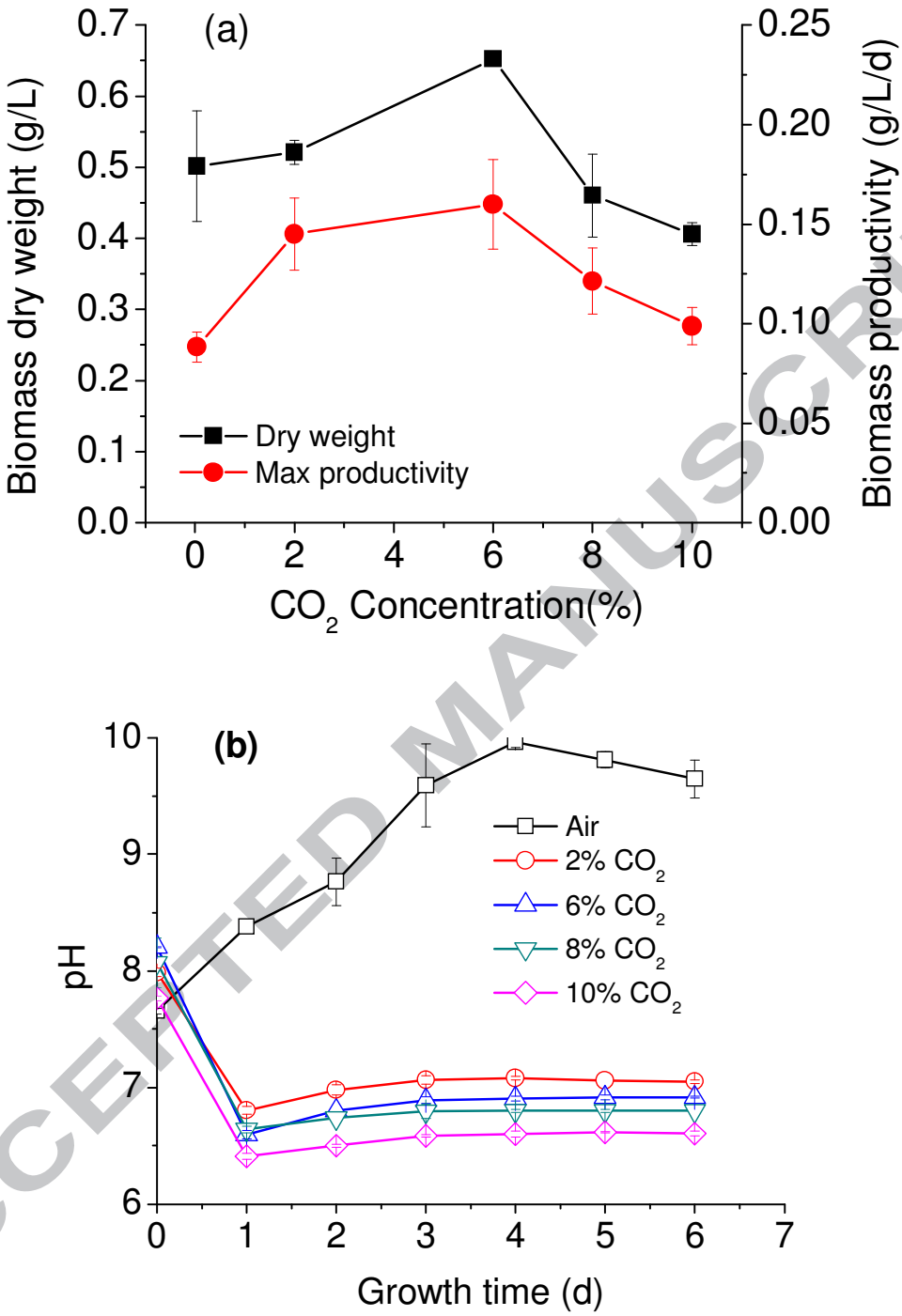

Fig.2. Growth curves of *Haematococcus pluvialis* mutant (a) and dynamic pH values of cultures (b) with various CO_2 concentrations

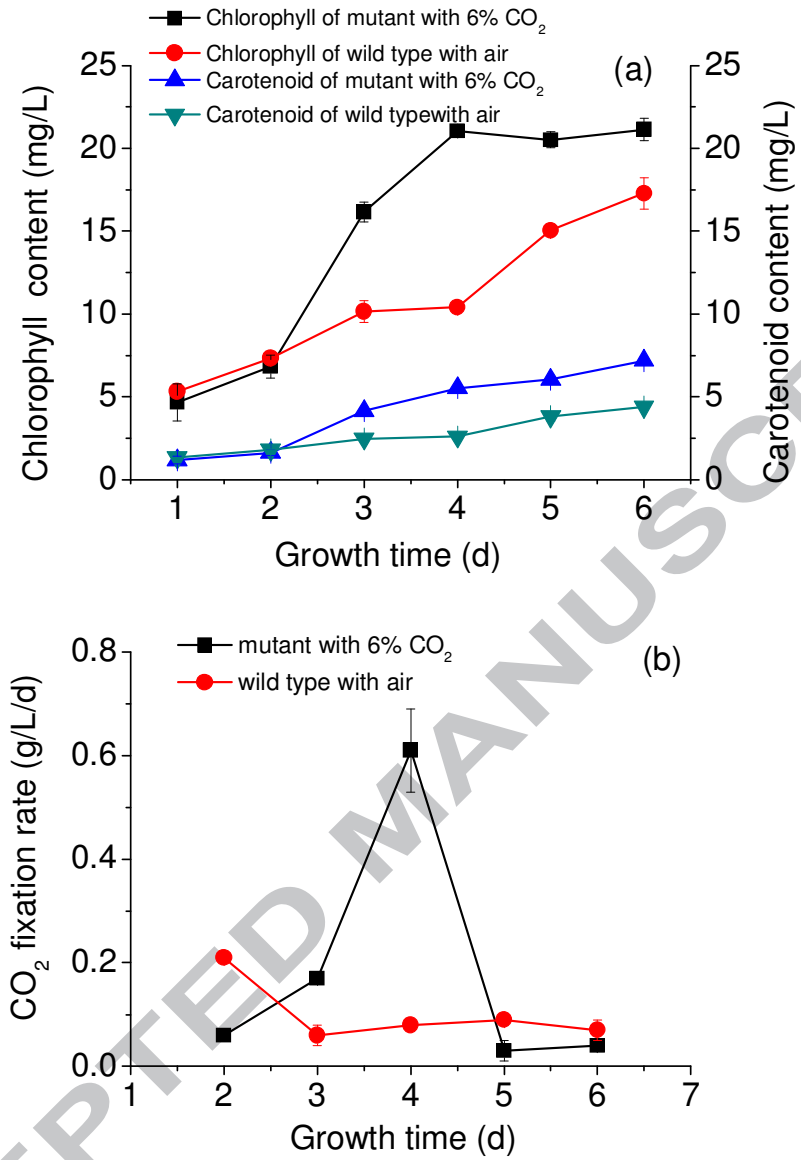

Fig.3.Comparison of *Haematococcus pluvialis* mutant with 6% CO_2 and the wild type with air in (a) chlorophyll and carotenoid contents, and (b) CO_2 fixation rate

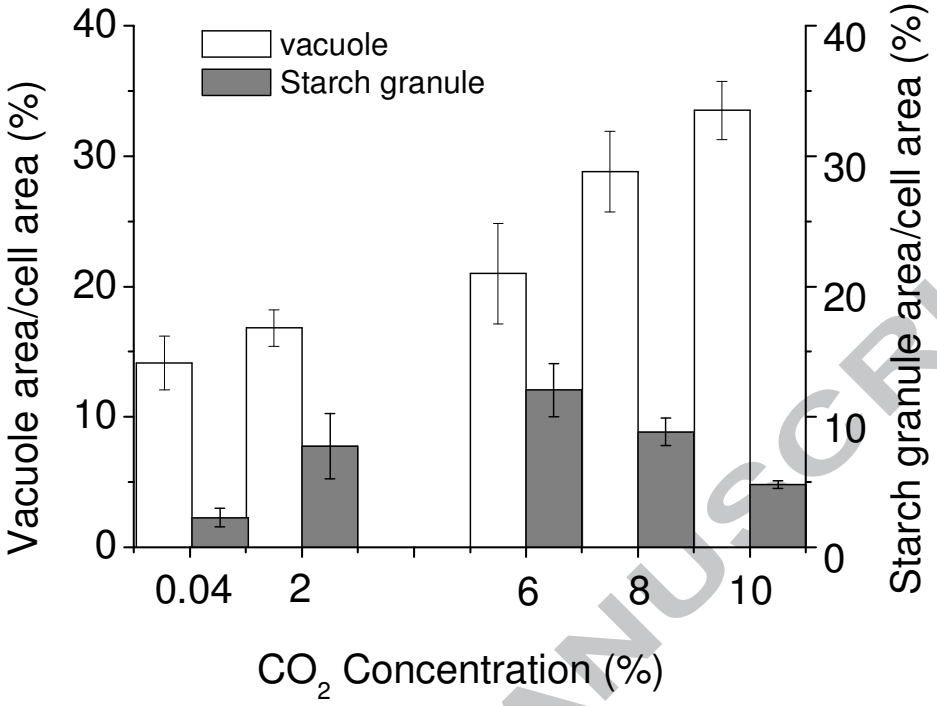

Fig.4. Cell ultrastructures (vacuole and starch granule) of *Haematococcus pluvialis* mutant cultured with various CO_2 concentrations based on TEM images

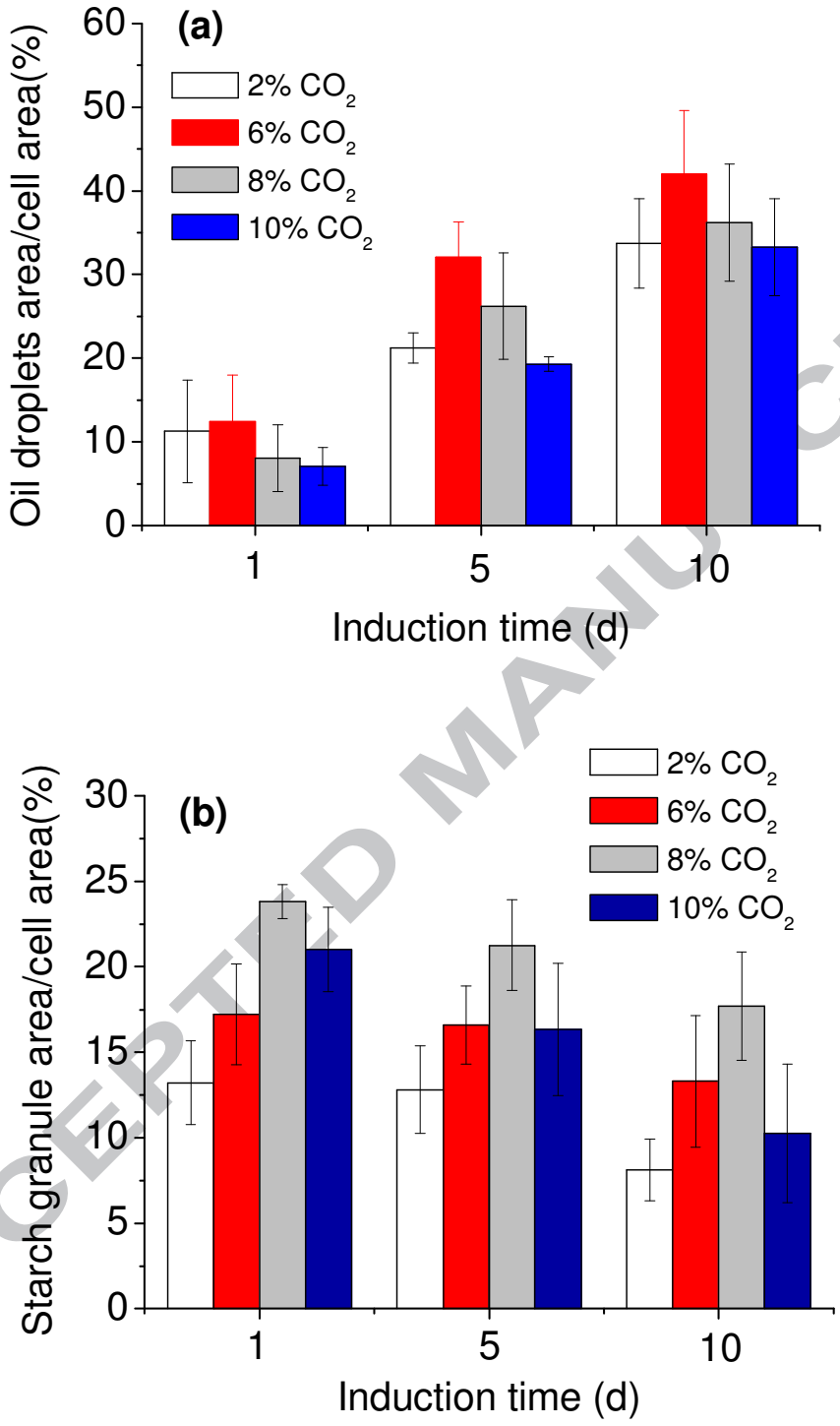

Fig.5. Cell ultrastructures (oil droplet (a) and starch granule (b)) of *Haematococcus pluvialis* mutant induced with high concentrations of CO_2 stress (Light=45 μmol photons $m^{-2}s^{-1}$) based on TEM images

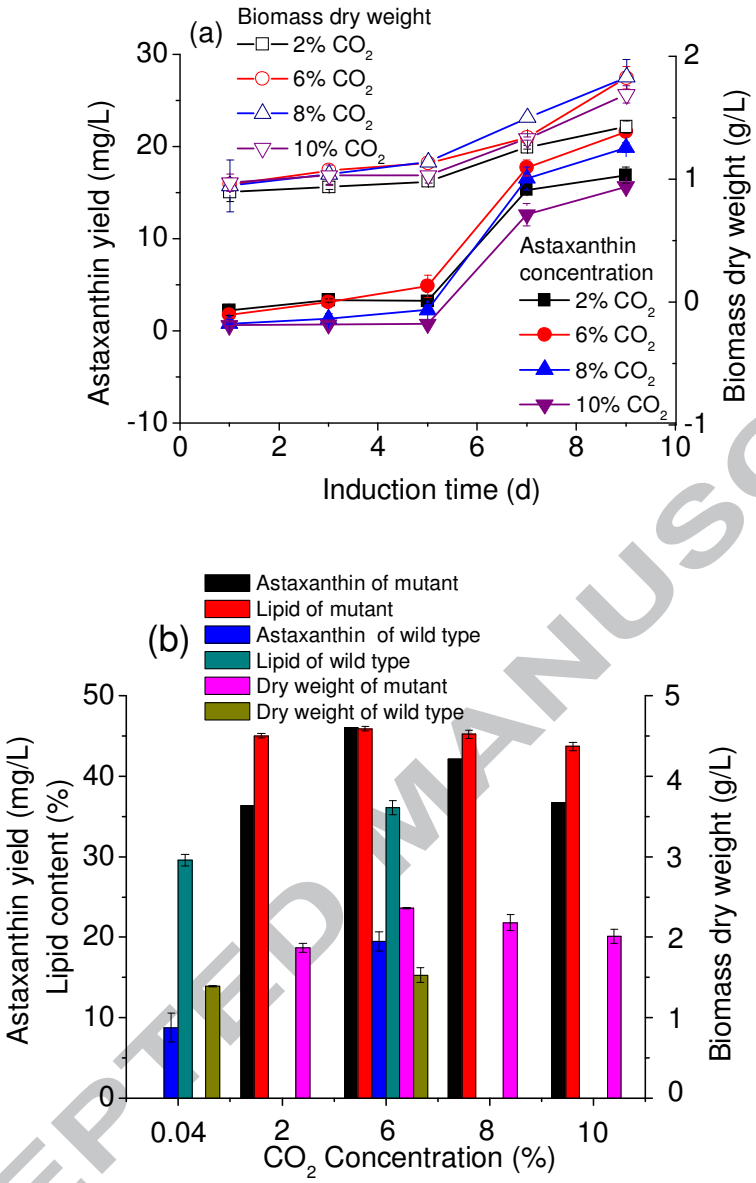

Fig.6. Biomass dry weight, astaxanthin yield and lipid content of *Haematococcus pluvialis* mutant induced by high concentrations of CO_2 stress with (a) low Light of 45 μmol photons m^{-2} s^{-1} and (b) high light of 108 μmol photons $m^{-2}s^{-1}$

>Fast-growing *Haematococcus pluvialis* mutant was obtained with ^{60}Co-γ irradiation

> The highest biomass and astaxanthin yields of mutant were obtained with 6% CO_2

> Astaxanthin and lipid yields of mutant with 6% CO_2 were higher than the wild type