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The Optimal Growth Conditions for the Biomass Production of *Isochrysis galbana* and the Effects That Phosphorus, Zn^{2+}, CO_2, and Light Intensity Have on the Biochemical Composition of *Isochrysis galbana* and the Activity of Extracellular CA

Sun Yingying[1] and Wang Changhai[2]*

[1] School of Ocean, Huaihai Institute of Technology, Lianyungang 222005, China
[2] School of Ocean, Yantai University, Yantai 264005, China

Abstract The effects of phosphorus, Zn^{2+}, CO_2, and light intensity on growth, biochemical composition, and the activity of extracellular carbonic anhydrase (CA) in *Isochrysis galbana* were investigated. A significant change was observed when the concentration of phosphorus in the medium was increased from 5 μmol/L to 1000 μmol/L affecting *I. galbana's* cell density, biochemical composition, and the activity of extracellular CA. Phosphorous concentration of 50 μmol/L to 500 μmol/L was optimal for this microalgae. The Zn^{2+} concentration at 10 μmol/L was essential to maintain optimal growth of the cells, but a higher concentration of Zn^{2+} (≥ 1000 μmol/L) inhibited the growth of *I. galbana*. High CO_2 concentrations (43.75 mL/L) significantly increased the cell densities compared to low CO_2 concentrations (0.35 mL/L). However, the activity of extracellular CA decreased significantly with an increasing concentration of CO_2. The activity of extracellular CA at a CO_2 concentration of 43.75 mL/L was approximately 1/6 of the activity when the CO_2 concentration was at 0.35 mL/L CO_2. Light intensity from 4.0 mW/cm^2 to 5.6 mW/cm^2 was beneficial for the growth, biochemical composition and the activity of extracellular CA. The lower and higher light intensity was restrictive for growth and changed its biochemical composition and the activity of extracellular CA. These results indicate that phosphorus, Zn^{2+}, CO_2, and light intensity are important factors that impact growth, biochemical composition and the activity of extracellular CA in *I. galbana*.

Keywords: Isochrysis galban, *extracellular carbonic anhydrase, light intensity, phosphorus, Zn^{2+}*

INTRODUCTION

Isochrysis galbana, a marine microalga, is of substantial interest in aquaculture for its good nutritive characteristics especially to mollusk larvae, fish, and crustaceans in the early stages of growth [1]. As aquaculture feed, the biochemical composition of the microalgae plays an important role in their nutritional value. It is well known that microalgae show considerable metabolic flexibility in response to changes in environmental factors and their biochemical composition also can be affected by the cultural conditions such as nutrient, irradiance, and temperature [2-4].

The effects of nutrient level and light intensity on the growth and biochemical composition have been established for some species of microalgae such as *Ankistrodesmus* [5], *Chlorella* [6], and *Chaetoceros mulleri* [7]. Fidalgo *et al*. reported that a nitrogen source can affect the lipid composition and fatty acid profile in *I. galbana* [8]. When nitrogen, silicon or light intensity is limited, the contents or composition of proteins, carbohydrates, pigments, lipids, and fatty acids also are changed in the cells of *I. galbana* [2,9].

In some marine microalgae, the extracellular carbonic an-

***Corresponding author**
Tel: +86-535-6706288 Fax: +86-535-6706299
e-mail: chwang2001@sina.com

hydrase (CA) was in the periplasmic space outside the plasma membrane or attached to the cell wall [10-12]. Two functions of extracellular CA have been proposed [13]: (1) it allows the cells to use C_i (dissolved inorganic carbon) at alkaline pH values *via* indirect acquisition of HCO_3^- by conversion of HCO_3^- to CO_2 which enters into the cell by diffusion or by active transport and (2) supply of HCO_3^- for direct uptake over the plasma membrane through hydration of CO_2. Some marine microalgae, *e.g. Amphidinium carterae* and *Prorocentrum minimum* have CA, but other marine microalgae, *e.g. Chaetoceros compressus* and *Glenodinium foliaceum* do not [14]. And, some marine phytoplankton which has the ability to use HCO_3^- might have a competitive advantage over those using CO_2 exclusively [15]. Some researches found that the activity of extracellular CA could be induced by some external factors, *e.g.* the concentration of free CO_2, pH, light, nitrogen concentration, and so on [16-18]; however, the interaction between environmental factors such as nutrients and irradiance and the activity of extracellular CA in *I. galbana* has not been elucidated.

Although *I. galbana* has been cultured successfully indoors and outdoors [19], it has not been used extensively because of difficulties in achieving high density cultures resulting in unreliable algal diet production [20]. In this study, we found the optimal conditions for phosphorus, Zn^{2+}, CO_2, and light intensity for the growth and biomass production of *I. galbana*. We observed the effects that phosphorus, Zn^{2+}, CO_2, and light intensity had on the biochemical composition of *Isochrysis galbana.* Finally we looked at its respective interaction between environmental factors and the activity of extracellular CA.

MATERIALS AND METHODS

Algal Species and Culture Conditions

The marine microalgae *I. galbana* was a gift from Marine Microalgae Research Center, Ocean University of China and cultured in Erlenmeyer flasks with f/2 medium [21]. Cultures were incubated for 10 days at 23°C and illuminated with fluorescent lamps in a 16/8 dark/light cycle, at an irradiance level of 4.0 mW/cm^2. Experiments were carried out in triplicate.

Phosphorus Concentration

All of the nutrients were prepared according to the recipe for f/2 medium with the exception of phosphorus. 5, 50, 100, 500, and 1,000 μmol/L of phosphorus were added to the 1,000 mL flasks containing 800 mL of phosphorus-free f/2 medium with an initial cell density of 0.88×10^7 cells/mL, respectively.

Zn^{2+} Concentration

Zn^{2+} was added in the form of zinc nitrate [$Zn(NO_3)_2$]. 10, 100, 1,000, and 10,000 μmol/L of Zn^{2+} were added to 1,000 mL Erlenmeyer flasks and diluted with 800 mL of Zn^{2+}-free f/2 medium. Then *I. galbana* was inoculated into them with an initial cell density of 0.88×10^7 cells/mL and the medium without Zn^{2+} was the control. Five milliliters samples from the cultures were assayed every two days to determine cell density and chlorophyll, protein and polysaccharide content. After 10 days, cells were harvested to determine extracellular CA activity.

CO_2 Concentration

Bottles containing 800 mL of minimal medium were vigorously aerated with oil-free compressed air containing 0.35, 26.25, and 43.75 mL/L CO_2 respectively and the concentration of CO_2 was controlled by a CO_2 meter. The medium and culture conditions are described elsewhere [21]. After 10 days, the cell density and the activity of extracellular CA were determined.

Light Intensity

The cells cultures were inoculated to an initial cell density of 0.69×10^7 cells/mL in 800 mL Erlenmeyer flasks containing f/2 medium, aerated with air, and irradiated by different light intensities for 10 days.

Cell Assay for Growth and Analysis of Biochemical Composition

0.1 mL samples were taken from cultures every two days and the cells were counted under BX-202B inverted microscope by a haemacytometer.

Referring to the method of Jensen [22], samples were collected on glass filters (Whatman GF/F). Then the filter was transferred to a 5 mL centrifuge tube with acetone and kept overnight at 4°C. The pellets were extracted again until colorless. Measurements were performed with a spectrophotometer and the equations given by Jensen were used to calculate the chlorophyll concentration. Cell suspensions (3 mL) were centrifuged at $5,000 \times g$ for 10 min. The pellets were resuspended in 3 mL potassium phosphate buffer (pH 7.8), sonicated for 3 min, and centrifuged at $5,000 \times g$ for 10 min. An aliquot of the extract was used to determine the protein concentration by the Bradford method utilizing bovine serum albumin as a standard [23]. The concentration of polysaccharide was assayed according to the method of Wang and Sun [24].

The Activity of Extracellular CA Assay

The activity of extracellular CA was measured by an electrometric method as described previously by Wilber and Anderson [25]. Intact cells were harvested by centrifugation at $5,000 \times g$ for 10 min, washed once with buffered veronal seawater at pH 8.2, and resuspended in 20 mmol/L buffered veronal seawater. 0.5 mL of cell sample (pH 8.2) was added to 4 mL of buffer and mixed. After the addition of 2 mL CO_2

Table 1. Effect of phosphorus concentrations on cell growth, biochemical synthesis, and the activity of extracellular CA in *I. galbana*

Concentrations of P (μmol/L)	Cell density ($\times 10^7$ cells/mL)	Protein (mg/g)	Polysaccharide (mg/g)	Chlorophyll (mg/g)	Extracellular CA (EU $\times 10^{-7}$ cells)
5	0.52 ± 0.03	116 ± 52	134 ± 58	12.3 ± 3.48	3.30 ± 0.97
50	1.26 ± 0.10	345 ± 32	210 ± 18	15.9 ± 3.23	40.1 ± 1.28
100	1.37 ± 0.10	301 ± 25	328 ± 29	32.4 ± 2.55	26.8 ± 1.63
500	1.69 ± 0.22	269 ± 36	244 ± 32	21.1 ± 1.37	25.0 ± 1.26
1000	1.42 ± 0.16	241 ± 22	201 ± 51	16.8 ± 2.41	21.8 ± 1.72

saturated icy pure water, the drift from pH 8.2 to 7.0 was observed over a period of time. In all the assays, the same cell density of 1.0×10^7 cells/mL was used. Enzyme units were calculated using the following equation: EU = 10 × (T_0/T-1). Where T_0 and T represent the times required for the reaction in the absence and presence of the sample, respectively.

Statistical Analysis

The data were statistically analyzed using the SPSS 12.0 software package by one-way ANOVA. Significant differences between two means were determined by Tukey's test. *P*-values < 0.05 were regarded as significant.

RESULTS

Effect of Phosphorus Concentration on the Growth, Biochemical Composition, and the Activity of Extracellular CA

Under the different phosphorus concentrations, the growth rates of *I. galbana* were varied and had significant differences among them ($P < 0.05$). With different concentrations of phosphorus by day 10, the maximum instantaneous growth rate was 0.26 d^{-1} for 5 μmol/L, 0.40 d^{-1} for 50 μmol/L, 0.41 d^{-1} for 100 μmol/L, 0.45 d^{-1} for 500 μmol/L, and 0.42 d^{-1} for 1,000 μmol/L of phosphorus, respectively. When the phosphorus concentration was 500 μmol/L, *I. galbana* reached the highest cell density with a significant difference of $P < 0.05$ (Table 1).

The activity of extracellular CA and the biochemical composition of *I. galbana* were both affected by the concentration of phosphorus (Table 1). Under the different phosphorus concentrations, the activity of extracellular CA, and the concentration of polysaccharides, chlorophyll, and the total proteins in the cells had a significant difference ($P < 0.05$). The differences between the concentrations of polysaccharides and chlorophyll in the cells were similar. When the phosphorus concentration was 100 μmol/L, the concentration of polysaccharides and chlorophyll in the cells were the highest, but the concentration of polysaccharides and chlorophyll in the cells began to decrease when the concentration of phosphorus was over 100 μmol/L. The total proteins in the cells were about 2~3 folds higher when the phosphorus concentration in the medium was over 50 μmol/L rather than when it was 5 μmol/L. At a concentration of 50 μmol/L, the activity of extracellular CA achieved its highest point which was about 12 folds higher than when the concentration was 5 μmol/L. Although the activity of extracellular CA decreased slowly when the phosphorus concentration increased from 50 μmol/L to 1,000 μmol/L, it was still about 7 folds higher than when the concentration was 5 μmol/L.

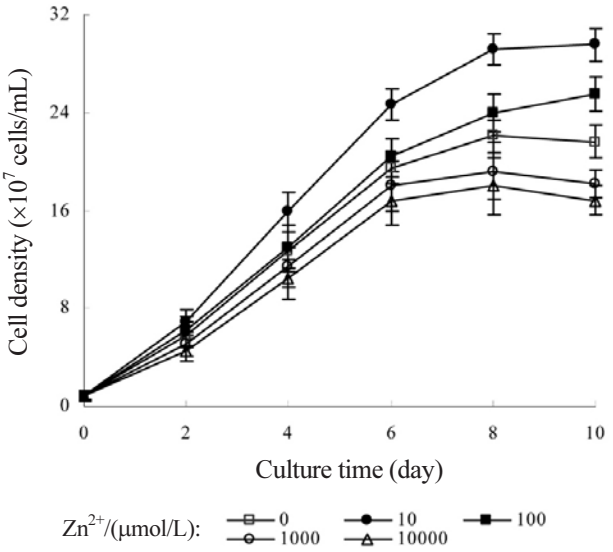

Fig. 1. Effect of Zn^{2+} concentration on the growth of *I. galbana*.

Effect of Zn^{2+} on the Growth, Biochemical Composition, and the Activity of Extracellular CA

As shown in Fig. 1, the cell densities between the control and Zn^{2+}-exposed cultures had significant differences ($P < 0.05$). The cell densities increased significantly ($P < 0.05$) under low Zn^{2+} concentrations (10~100 μmol/L) which was still higher than that of the control. When Zn^{2+} concentration was over 1,000 μmol/L, the growth was inhibited and the cell density was significantly ($P < 0.05$) less than that of the control.

After 10 days of Zn^{2+} exposure, the chlorophyll in the cells was affected more severely than that of the proteins or polysaccharides in the cells (Figs. 2~4). In low Zn^{2+} (10 μmol/L) cultures, the chlorophyll in the cells was signifycantly ($P < 0.05$) higher than that of the control; however,

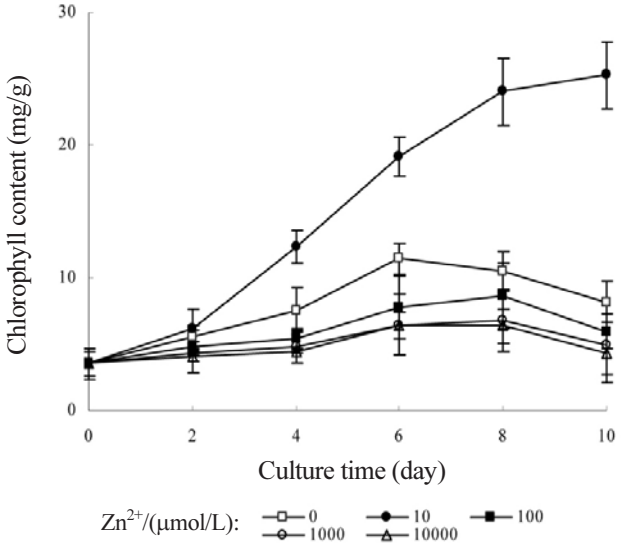

Fig. 2. Effect of Zn^{2+} concentration in medium on the chlorophyll concentration in *I. galbana*.

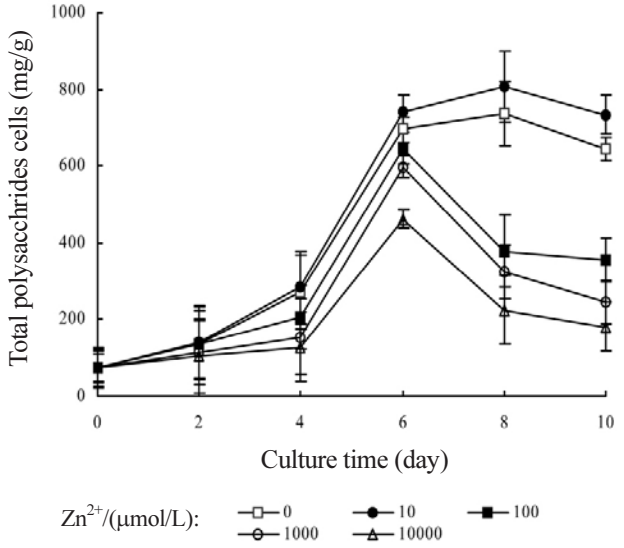

Fig. 4. Effect of Zn^{2+} concentration on the total polysaccharides concentration in *I. galbana*.

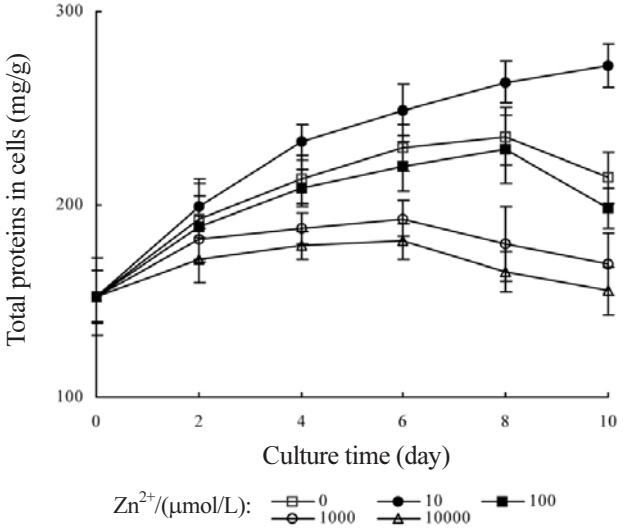

Fig. 3. Effect of Zn^{2+} concentration in medium on the total proteins concentration in *I. galbana*.

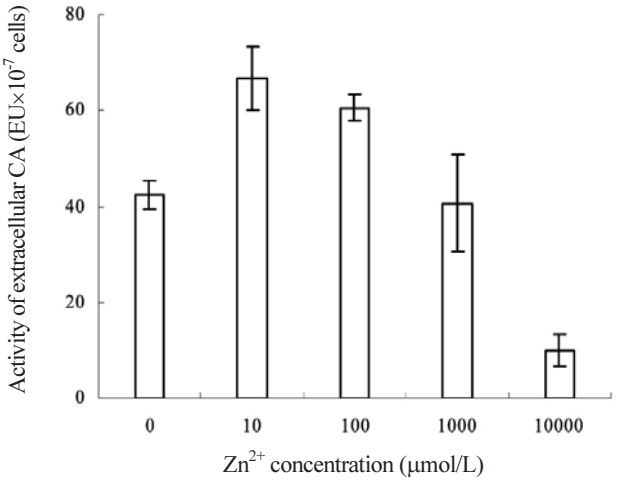

Fig. 5. Effect of Zn^{2+} concentration on the activity of extracellular CA in *I. galbana*.

the chlorophyll in the cells was significantly ($P < 0.05$) lower than that of the control when Zn^{2+} concentrations were 1,000 μmol/L and 10,000 μmol/L. A Zn^{2+} concentration of 10 μmol/L provoked a significant ($P < 0.05$) increase in total cell protein concentration. An adverse effect was observed when the Zn^{2+} concentration was over 100 μmol/L with the total proteins in the cells decreasing significantly ($P < 0.05$) with increasing Zn^{2+} exposure levels. Within a Zn^{2+} concentration range from 100 μmol/L to 10,000 μmol/L, the concentration of polysaccharides in the cells decreased drastically with increasing Zn^{2+} concentrations, but the concentration of polysaccharides in the cells was significantly ($P < 0.05$) higher than that of the control when the concentration of Zn^{2+} was at 10 μmol/L.

With increasing Zn^{2+} concentrations from 0 to 100 μmol/L, the activity of extracellular CA increased from 42.45 EU × 10^{-7} cells to 60.53 EU × 10^{-7} cells (Fig. 5). The activity of extracellular CA was significantly ($P < 0.05$) higher than that of the control when the concentration of Zn^{2+} was between 10 μmol/L and 100 μmol/L and reached the highest value of 66.64 EU × 10^{-7} cells at a Zn^{2+} concentration of 10 μmol/L. With increasing Zn^{2+} concentrations from 1,000 μmol/L to 10,000 μmol/L, the activity of extracellular CA was inhibited strongly by Zn^{2+} and decreased rapidly from 40.70 EU × 10^{-7} cells to 9.89 EU × 10^{-7} cells.

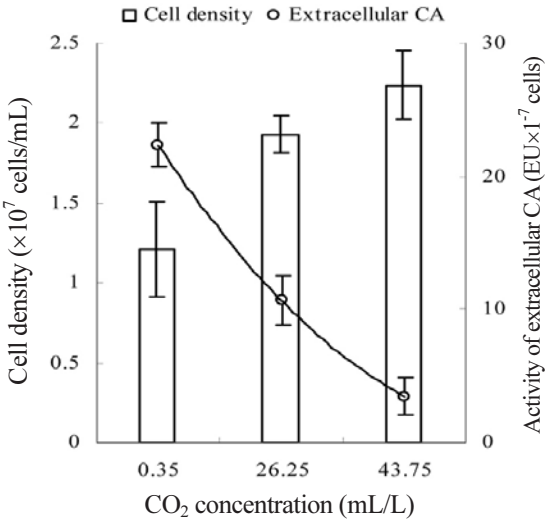

Fig. 6. Effect of CO_2 concentration on cell growth and activity of extracellular CA in *I. galbana*.

Effect of CO_2 Concentration on the Growth and the Activity of Extracellular CA

In order to characterize the growth and regulation of extracellular CA, *I. galbana* cells were cultured under various concentrations of CO_2. As shown in Fig. 6 when the concentration of CO_2 increased from 0.35 mL/L to 43.75 mL/L, cell growth increased significantly ($P < 0.05$); however, the activity of extracellular CA declined significantly ($P < 0.05$) with increasing CO_2 concentrations. When the concentration of CO_2 was at 0.35 mL/L, the activity of extracellular CA was about 6 folds higher than when the concentration was at 43.75 mL/L.

Effect of Light Intensity on the Growth, Biochemical Composition, and the Activity of Extracellular CA

Table 2 shows the influence of light intensity on growth, biochemical composition, and the activity of extracellular CA. When light intensity was increased from 3.2 mW/cm^2 to 4.8 mW/cm^2, the cell density increased. When light intensity was over 4.8 mW/cm^2, the cell density began to decrease. The results clearly show that a light intensity of 4.0 mW/cm^2 to 5.6 mW/cm^2 is optimal for the growth of *I. galbana* and that lower or higher light intensities was restrictive for the growth of *I. galbana*.

With increasing light intensity, the total proteins in the cells decreased slowly and the concentration of polysaccharides significantly ($P < 0.05$) increased in the cells. When light intensity was increased from 3.2 mW/cm^2 to 6.4 mW/cm^2, the total proteins in the cells decreased only about 40%, but a 3.22 fold increase in total polysaccharides in the cells was observed. The chlorophyll concentration in the cells increased when light intensity increased from 3.2 mW/cm^2 to 4.8 mW/cm^2 and peaked at a maximum value of 21.98 mg/g. It then decreased quickly with increasing light intensity.

At a light intensity of 4.8 mW/cm^2, the activity of extracellular CA reached its highest point which was about 3 folds higher than when the light intensity was at 3.2 mW/cm^2. The activity of extracellular CA decreased with increasing light intensity (4.8 mW/cm^2 to 6.4 mW/cm^2); however, the activity of extracellular CA was still higher than when the light intensity was at 3.2 mW/cm^2.

DISCUSSION

Phosphorus plays a significant role in most cellular processes, especially those involved in generating and transforming metabolic energy. Kuhl found that in phosphate-deficient *Ankistrodesmus* cultures, dry weight, cell division, photosynthetic oxygen production, and chlorophyll synthesis were inhibited under the conditions of phosphorus starvation [5]. The inter-relationships between phosphorus availability and the basic physiological reactions such as the growth, reproduction, photosynthesis, or respiration have also been reported in other microalgae [26,27]. Our research results confirmed that not only the growth, but the biochemical composition (such as chlorophyll, protein, and polysaccharide) and the activity of extracellular CA in *I. galbana* can be affected by phosphorus. Sufficient phosphorus in the medium was beneficial for optimal growth and the expression of the activity of extracellular CA.

Our research shows that the cell density, chlorophyll concentration, the total proteins in the cell, concentration of polysaccharides, and the activity of the extracellular CA were lower under Zn^{2+} starvation in the cells. And the concentration of Zn^{2+} at 10 μmol/L was essential to keep optimal growth of *I. galbana*; whereas, high concentrations of Zn^{2+} ($\geq$ 1,000 μmol/L) did inhibit the growth of *I. galbana*. The results were similar to our previous research in which we found that Zn^{2+} was an essential micronutrient for *I. galbana*;

Table 2. Effect of light intensity on cell growth, biochemical synthesis, and the activity of extracellular CA in *I. galbana*

Light intensity (mW/cm^2)	Cell density (× 10^7 cells/mL)	Protein (mg/g)	Polysaccharide (mg/g)	Chlorophyll (mg/g)	Extracellular CA (EU × 10^{-7} cells)
3.2	1.28 ± 0.06	183 ± 39	128 ± 24	14.84 ± 0.98	6.08 ± 0.52
4.0	1.71 ± 0.11	178 ± 72	217 ± 35	19.55 ± 0.83	6.88 ± 0.48
4.8	2.12 ± 0.20	166 ± 14	241 ± 96	21.98 ± 1.16	19.03 ± 0.55
5.6	1.89 ± 0.14	164 ± 25	383 ± 87	12.08 ± 0.54	12.99 ± 0.65
6.4	1.22 ± 0.08	136 ± 44	412 ± 31	11.17 ± 1.07	10.71 ± 0.37

however, Zn^{2+} could be toxic at higher concentrations and cause the microalgal cell death [28]. CA is a Zn^{2+} containing metalloenzyme and may provide high concentrations of CO_2 to Rubisco during CO_2 fixation. Our data suggests that Zn^{2+} may affect photosynthesis by acting on CA. Similar results were reported in other microalgae [29,30]. Therefore, it is clear that the balance of available nutrients for optimal microalgal growth was disturbed by higher concentrations of Zn^{2+} from 1,000 μmol/L to 10,000 μmol/L in the media.

CO_2 is one of the most important nutrients when culturing microalgae. The results showed that high CO_2 concentrations (26.25 mL/L and 43.75 mL/L) significantly increased the cell density of *I. galbana* when compared to low concentrations of CO_2 (0.35 mL/L). Aerating the cultures with CO_2 improved the saturation of CO_2[DK1], helped control the pH in culture, and increased the growth rate and the maximum cell density. The activity of extracellular CA in *I. galbana* decreased significantly with increasing concentrations of CO_2 and the activity of extracellular CA at a CO_2 concentration of 43.75 mL/L was only about 1/6 fold than when the CO_2 concentration was 0.35 mL/L.

Light intensity did influence biochemical composition and activities of some enzymes such as CA in *I. galbana*. Li *et al*. reported that the percentage of protein and carbohydrate in *Phaeodactylum tricornutun* decreased with increasing light intensity [31]. Villand *et al*. found that the expression of the mitochondrial CA genes depends on light intensity [32]. In our experiments, the range of light intensity from 4.8 mW/cm^2 to 5.6 mW/cm^2 was beneficial for cell growth, chlorophyll, protein, and polysaccharide biosynthesis and for the activity of extracellular CA. Lower or higher light intensity was restrictive for cell growth, and decreased the total concentrations of chlorophyll, proteins, polysaccharides, and decreased the activity of extracellular CA in *I. galbana*.

CONCLUSION

The concentration of phosphorus and Zn^{2+} can significantly impact cell growth, concentrations of polysaccharide, chlorophyll, and protein, and the activity of extracellular CA in *I. galbana*. High CO_2 concentrations significantly increased the cell density in culture when compared to low CO_2 concentrations; however, the activity of extracellular CA decreased significantly with increasing concentrations of CO_2. In addition when the light intensity increased from 3.2 mW/cm^2 to 4.8 mW/cm^2, the cell density in culture, chlorophyll concentration, and the activity of extracellular CA reached its maximum value. Therefore, it is possible to adjust the growth and adjust the output of biochemical pathways and the activity of extracellular CA in *I. galbana* by controlling the concentrations of phosphorus, Zn^{2+}, CO_2, and the light intensity. A high cell biomass, rich biochemical synthesis, and high activity of extracellular CA can be obtained.

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