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Low-molecular-mass organic acid and lipid responses of *Isochrysis galbana* Parke to high temperature stress during the entire growth stage

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ABSTRACT

Isochrysis galbana is an important food source in aquaculture especially in the rearing of bivalve mollusks and usually cultured in summer, when water temperatures in breeding pools may reach 35 °C. However, the impact of high temperature stress on the growth and metabolism of *I. galbana* is poorly understood. In this study, the changes of total superoxide dismutase (T-SOD) activity, organic acid, and lipid levels were analyzed at different growth stages when *I. galbana* Parke was cultured under normal temperature (20 °C) and extreme high temperature (35 °C), respectively. The result showed that T-SOD activity reached the highest level at exponential phase at both temperatures but exhibited lower level during growth at 35 °C. The lower content of citric acid and α-ketoglutaric acid during late stationary phase indicated that tricarboxylic acid (TCA) cycle, amino acid metabolism, and the synthesis of fatty acids may severely be damaged under high temperature stress. The upregulation of monogalactosyldiacylglycerol (MGDG), diacylglycerylcarboxyhydroxymethylcholine (DGCC), diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS), triacylglycerol (TAG), and lyso-lipids was observed from adaptation phase to early stationary phase. But downregulation of glyceroglycolipids (MGDG, DGDG, and SQDG), DGCC, and DGTS were found between early stationary phase and late stationary phase at high temperature. Besides, the level of DHA decreased under high temperature stress, revealing that the nutritional value of *I. galbana* Parke had been reduced.

This is the first report addressing metabolic responses of *I. galbana* Parke to high temperature stress combining T-SOD activity, organic acid, and lipid analysis, and the results demonstrated that the extreme high temperature (35 °C) will not only suppress the activity of T-SOD and the formation of organic acids in the TCA cycle and photorespiration, but also influence the lipid metabolism in *I. galbana* Parke. This study provided a useful reference value for the cultivation of *I. galbana* under high temperature condition.

1. Introduction

Microalgae are regarded a useful feed in aquaculture and play an important role in the growth and development of the fed animal. *I. galbana* is known to have good nutritional value due to its high content of long chain polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) [1]. Therefore, *I. galbana* has been widely used as a food source in all growth stages of bivalves, and in the larvae of zooplankton, crustaceans, and fish [2]. In the artificial rearing of many bivalve mollusks, especially after they are just hatched, *I. galbana* has been considered the essential diet. For example, *I. galbana* was found to be a good single-species diet for greatest shell growth and highest survival rates of juvenile razor clams [3]. *I. galbana* can promote shell growth more significantly than other algal diets during breeding of the spat and juvenile pipi clam [4]. As a result, more and more attention has been paid to the growth and metabolism of *I. galbana* for its potential application in aquaculture.

Abbreviations: ACL, the average chain length; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DGCC, diacylglycerylcarboxyhydroxymethylcholine; DGDG, igalactosyldiacylglycerol; DGTS, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine; DHA, docosahexaenoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; H_2O_2, hydrogen peroxide; MGDG, monogalactosyldiacylglycerol; OPLS-DA, orthogonal projection to latent structures with discriminant analysis; o-BHA, o-benzylhydroxylamine; PBS, phosphate buffered saline; PCA, principal component analysis; PLS-DA, projection to latent structures with discriminant analysis; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SQDG, sulfoquinovosyldiacylglycerol; SRM, selected reaction monitoring; TAG, triacylglycerol; TCA, tricarboxylic acid; T-SOD, total superoxide dismutase; UI, unsaturation index; VIP, variable important in the projection

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Recently, many studies focus on the influence of environmental factors on the biochemical composition and content of microalgae. For example, Lin et al. studied the effect of different components of medium on the metabolism in *I. galbana* cultivation to achieve high amounts of polyunsaturated fatty acids [5]. There are also reports studying the impacts of light conditions on growth of *I. galbana*, such as light strength and light frequency [6,7]. Different salinities were tested to evaluate the growth, chlorophyll *a* and protein content of *I. galbana* [8]. The metabolism of *I. galbana* in response to temperature change was investigated extensively. Durmaz et al. reported that the growth and biochemical composition of *I. galbana* were subject to the temperature changes (18 °C and 26 °C) [9]. Zhu et al. found that carbohydrate and protein content of *I. galbana* varied at two culture temperatures (15 °C and 30 °C) and growth phase, and lipid was accumulated in the stationary growth phase [10]. Temperature was known to be important for cell cultures by influencing the structure of cell components and metabolic reaction rate [11]. In recent years, temperature was continually increasing because of global warming and gradually became a serious threat to the growth and development of organisms. In China, summer is the season for the artificial hatchery of many bivalve mollusks, such as *Tegillarca granosa*, *Scapharca subcrenata*, *Meretrix lyrata*, *Cyclina sinensis* and *Moerella iridescens*. However, *I. galbana* culture in summer is very difficult, probably because the water temperature at noon in summer can reach 35 °C under constant sun exposure, which significantly disturbs the normal cellular homeostasis of *I. galbana* and leads to its abnormal growth, metabolism, and even death. As a result, the aquaculture industry suffers great economic loss. Therefore, it is of great interest to evaluate the changes of some important metabolites in response to high temperature stress in hope of understanding how high temperature affects its growth and metabolism. Considering the growth and metabolism of microalgae may vary at different growth phases [10], we investigated the effect of both temperature and growth phase on the changes of metabolism simultaneously.

Reactive oxygen species (ROS) can oxidize molecules in chloroplasts such as D1 protein in photosystem and thiol enzymes in the Calvin-Benson cycle to inhibit some reaction of photosynthesis [12]. A number of studies demonstrated that ROS-scavenging mechanisms play an important role in protecting organism against high temperature stress [13,14]. Superoxide dismutase (SOD), the first line of cellular defense against oxidative damage, can catalyze the conversion of ROS into hydrogen peroxide (H_2O_2) to alleviate the damaging effect [15]. Therefore, the SOD is closely related to the ROS production. It was reported that the variation of some organic acids content appeared to be correlated with photosynthesis, which can absorb sunlight through chlorophyll molecules in photosystems I (PSI) and II (PS II) [16,17]. The organic acids in the tricarboxylic acid (TCA) cycle not only are crucial in photosynthetic sucrose synthesis and cell growth, but are involved in other metabolic processes such as lipid and amino acid metabolism [18]. Considering that lipids are building the blocks of membranes, involved in energy storage and in some metabolic processes, lipids were extensively analyzed in the study, especially during acute and chronic stress [19–21]. Therefore, combined analysis of T-SOD activity, organic acid and lipid contents can effectively reflect how high temperature stress affects the growth and metabolism of *I. galbana*.

In this study, we investigated the metabolic changes of *I. galbana* Parke in response to high temperature stress using the hydroxylamine method, high-performance liquid chromatography coupled with tandem-mass spectrometry (HPLC/QQQ-MS), and ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS). The results revealed that extreme temperature (35 °C) would exacerbate harmful effects on the growth and metabolism of *I. galbana* Parke. Our finding not only can promote better understanding of the physiology and ecology of *I. galbana* under high temperature conditions, but also provide a useful reference value for its cultivation.

2. Material and methods

2.1. Microalgae culture and harvest

I. galbana Parke was obtained from the Marine Biotechnology Laboratory of Ningbo University and grown in NMB3 medium (KNO_3 100 mg/L, KH_2PO_4 10 mg/L, $MnSO_4{\cdot}H_2O$ 2.5 mg/L, $FeSO_4{\cdot}7H_2O$ 2.5 mg/L, EDTA-Na_2 10 mg/L, VB_1 6 μg/L, VB_{12} 0.05 μg/L) [3]. 12 cultures were kept at 20 °C (normal temperature) under 2200 lx light intensity of cool white fluorescent lamps with a 12:12 h L:D cycle. 2000 mL of cultures were collected on days 2 (adaptation phase), day 10 (exponential phase), day 18 (early stationary phase), and day 28 (late stationary phase). Another 12 cultures were maintained at 35 °C (extreme high temperature) at the same condition described above and collected on days 2 (adaptation phase), day 4 (exponential phase), day 8 (early stationary phase), and day 18 (late stationary phase). Then all cultures were centrifuged at 9000 rpm for 10 min at 4 °C and the pellets were freeze-dried. The experiments were performed in triplicates. The data is presented as mean ± standard deviation.

2.2. Total superoxide dismutase (T-SOD) activity assay

For T-SOD activity assay, 10 mg freeze-dried samples were homogenized with 800 μL of 0.1 mol/L phosphate buffered saline (PBS) followed by centrifugation at 3500 rpm for 10 min at 4 °C. The supernatants were collected and stored at 4 °C until assay. T-SOD activity was determined with the assay kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China). The unit of T-SOD activity was defined as the amount of enzyme that causes 50% inhibition of nitrite formation from hydroxylammonium in the presence of O_2^- generators. The protein concentration was measured by a protein detection kit purchased from TransGen Biotechnology Institute (Beijing, China). Bovine serum albumin (BSA) was used as the standard to plot the standard curve. The enzyme activities were presented as units (U) mg^{-1} protein.

2.3. Organic acid extraction and derivatization

200 μL mixed isotope internal standards solution (glycolic acid-$^{13}C_2$, L-malic acid-1-^{13}C, succinate acid-2,2,3,3,-d_4, fumaric acid-d_2, citric acid-2,2,4,4-d_4, *cis*-aconitic acid-$^{13}C_6$, and 2-α-ketoglutaric acid-1-^{13}C) and 800 μL water/acetonitrile mix (1:1, v/v) were added into 5 mg of the freeze-dried samples. The cells were homogenized and centrifuged at 12000 rpm for 10 min at 4 °C. Then the upper phase was dried in a rotary evaporator and dissolved in water. The derivatization method was adopted from Bo Tan et al. [22], but with some modifications. Briefly, the extracts were spiked with 50 μL of 1 mol/L o-benzylhydroxylamine (o-BHA) and 15 μL of 1 mol/L 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the pyridine buffer. After one-hour incubation at room temperature, 300 μL of ethyl acetate was added and the solution was shaken for 5 min. The pooled organic layers were dried with nitrogen at 40 °C after being extracted twice with ethyl acetate. Finally, the residual sample was dissolved in 1 mL of methanol/H_2O/formic acid (1:1:0.02, v/v/v) and filtered using a 0.22 μm ultrafiltration filter membrane (Millipore, Bedford, MA, USA). Before the HPLC-MS/MS analysis, the solution was diluted for optimal signal resolution in the mass spectrometer.

2.4. HPLC-MS analysis for organic acid

The analysis was performed using a Finnigan Surveyor and TSQ Quantum Access system (Thermo-Fisher Scientific, USA) with a Hypersil GOLD C18 analytical column (2.1 × 100 mm i.d., pore size 175A, particle size 3 μm, Thermo Fisher Scientific). 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used as the mobile phases. The gradient elution at a flow rate of 0.3 mL/min was as