

Article

The Impact of Cr(III) and Cr(VI) on Lipid Accumulation in *Chlorella pyrenoidosa*

Tianji Liu [†], Huawei Guo [†], Qing Yu, Yajun Wang, Huan Liu, Yanan Zeng, Yitong Wang ^{*✉}, Chunyu Liu ^{*} and Junguo Li

College of Metallurgy and Energy, North China University of Science and Technology, 21 Bohai Street, Tangshan 063210, China; liutianji@ncst.edu.cn (T.L.); ghw07021914@163.com (H.G.); qyu0688@gmail.com (Q.Y.); wangyj@ncst.edu.cn (Y.W.); lh18201127226@163.com (H.L.); zengyanan@ncst.edu.cn (Y.Z.); lijg99@163.com (J.L.)

^{*} Correspondence: wangyt@ncst.edu.cn (Y.W.); liucy@ncst.edu.cn (C.L.)

[†] These authors contributed equally to this manuscript.

Abstract: In this study, *Chlorella pyrenoidosa* was cultured with different concentrations of Cr(III) and Cr(VI), and the changes in biomass, lipid content, protein content and total sugar content of *Chlorella pyrenoidosa* under different culture conditions were detected. The effects of Cr(III) and Cr(VI) on the intracellular antioxidant system and related key enzymes of lipid synthesis were analyzed. The results showed that appropriate Cr(III) and Cr(VI) concentrations could make *Chlorella pyrenoidosa* regulate the contents of SOD, POD, ASA, CAT and GSH in cells, improve the enzyme activity of ACC and promote lipid synthesis. The aim was to investigate the mechanism of lipid synthesis by analyzing the distribution of Cr(III) and Cr(VI) in algal cells. The lipid content of *Chlorella pyrenoidosa* reached its highest level at 50.43 wt% and 38.00 wt% when both Cr(III) and Cr(VI) concentrations in the culture environment were 0.5 mg/L. These values were 1.37 and 1.03 times higher than the control group, respectively. The addition of appropriate Cr(III) and Cr(VI) concentrations can increase ME enzyme activity and decrease PEPC enzyme activity, shorten the biomass accumulation cycle and prolong the lipid synthesis cycle.

Keywords: *Chlorella pyrenoidosa*; chromium; enzyme activity; lipid; mechanism of lipid synthesis



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1. Introduction

There are three main forms of Cr in nature: one is the uncharged metallic form (Cr), another is the tri-valent form (Cr(III)) and the last is the six-valent form (Cr(VI)) [1]. In humans, Cr(III) is a micronutrient essential for glucose, lipid and protein metabolism. In contrast, Cr(VI) has no recognized biological function and is a potent carcinogen [2]. In the water, Cr exists as Cr(VI) in the body of fish and reaches the human body through the food chain [3]. The consumption of plants growing on contaminated soil will cause serious harm to human health due to the bioaccumulation of toxic heavy metals [4]. Cr(VI) is 100 times more poisonous than Cr(III), has high solubility, bioavailability, fluidity and biotoxicity and has been listed as one of the 20 most toxic pollutants [5]. Cr(VI) has a strong oxidizing ability and can change the expression of plant genes and proteins, thus disturbing plant metabolism. The U.S. Environmental Protection Agency demands that the amount of total chromium released into surface water should not exceed 2 mg/L and the level of Cr(VI) must not surpass 0.05 mg/L [6]. There are several common methods to address Cr, which primarily encompass physical, chemical and biological approaches [7]. The first two methods are easy to cause secondary pollution and cannot meet the national emission limits, which costs a lot and lacks efficiency. The biological method is a strategy that uses biological organisms such as bacteria, fungi, plants and algae to restore the environment to its original state. The biological processes include biosorption, bioaccumulation and biotransformation mechanisms for pollutant detoxification, which is a natural process [8]. In the case of bacteria and fungi, to promote their growth they need to supplement with

additional sources of nutrients [9]. The efficiency of the remediation of plants largely depends on the type of aquatic plants that accumulate heavy metals. The efficacy of plants depends upon a wide range of root systems, as they need a long amount of time to cultivate and produce aquatic plants [10]. Nevertheless, microalgae could potentially serve as an environmentally friendly method of remediation. Microalgae are photosynthetic organisms that can rapidly reproduce, use sunlight, water and carbon dioxide to convert into chemical energy and can produce biomass through the use of the nutrients present in wastewater while producing biomass that can be used to produce different value-added products [11], which can reduce additional nutrient requirements. Meanwhile, microalgae have excellent adsorption performance against Cr, which is an excellent candidate for removing Cr from wastewater. The excessive deposition of chromium in plant cells leads to alterations in enzyme activity, degradation of chlorophyll, reduction in biomass and disruption of the antioxidant defense system [12]. This applies to both Cr(III) and Cr(VI) which have varying levels of bioavailability, mobility and toxicity [13,14]. The removal of chromium by microalgae cells occurs not only through adsorption, but also through biological mechanisms. One example is the enzymatic reduction of Cr(VI) to Cr(III) as a means of alleviating its toxicity. When *Chlorella vulgaris* is under Cr(VI) stress will release Glutathione (GSH) which can reduce Cr(VI) to Cr(III). Additionally, most Cr(VI) is aggregated in organelles of microalgae cells [15]. Existing research focuses on the tolerance ability and removal ability of microalgae to Cr(VI). Lu et al. have studied *Chlorella vulgaris* in bioaccumulation of Cr(VI), where they found that the growth, chlorophyll content, oxidative damage and Cr(VI) removal rate under Cr(VI) stress were evaluated [16]. Shen et al. found that in the presence of added Cr (VI) and sodium acetate in *Botryococcus* sp. NJD-1 for Cr (VI) removal of 94.2% [6].

According to the above research, microalgae have been found to effectively remove Cr(VI). Some studies have shown that microalgae not only produce enough biomass and accumulate a significant amount of lipids under Cr stress, but also have the ability to bioremediate Cr(VI) within the toxic concentration limits for the human body (1 mg/L) [1]. Kafil et al. showed that *Chlorella* under Cr(VI) stress can change the content of fatty acid methyl esters in microalgae, thereby improving the quality of biodiesel [17].

Nevertheless, there is a lack of comprehensive research on the relationship between Cr and lipid accumulation in microalgae. Hence, the primary focus of this study is to assess the effects of Cr(III) and Cr(VI) on *Chlorella pyrenoidosa* biomass, lipid content, antioxidant system and key enzymes associated with lipid synthesis. Cr(III) and Cr(VI) were studied in valence transformation under the action of *Chlorella pyrenoidosa*. This study guides large-scale energy microalgae cultivation and high value-added utilization of Cr-containing waste.

2. Materials and Methods

The materials used in the experiment are presented in Section 2.1 and information on analytical methods and instrument parameters is shown in Sections 2.2 and 2.3.

2.1. Materials

The *Chlorella pyrenoidosa* used in the experiment was purchased from Nanjing Haiersi Biological Co., Ltd. (Nanjing, China). Table 1 lists the chemical reagents used for this study. Deionized water (pH: 5.83; ionization constant: 18.25 MΩ·cm) was from the ultrapure water system (Exceed-Ad-32, Chengdu Eco Water Treatment Equipment Co., Ltd., Chengdu, China). The ultraviolet/visible/near-infrared spectrophotometer used in the experiment came from Hitachi Manufacturing Co., LTD. (UH4150, Hitachi Manufacturing Co., Ltd., Tokyo, Japan).

Table 1. Chemical reagents used in study.

Reagents	Chemical Formula	Purity	Manufacturer	Location
Chromium trichloride hexahydrate	CrCl ₃ ·6H ₂ O	≥98.00%	Aladdin Reagent Co., Ltd.	Shanghai, China
Copper sulfate pentahydrate	CuSO ₄ ·5H ₂ O	≥99.00%		
Sodium carbonate	Na ₂ CO ₃	≥99.50%		
Dipotassium phosphate	K ₂ HPO ₄	≥99.00%		
Ammonium ferric citrate	C ₆ H ₁₁ FeNO ₇	≥99.00%, Fe: 20.50–22.50%		
Disodium dihydrate ethylenediamine tetraacetate	C ₁₀ H ₁₄ N ₂ Na ₂ O ₈ ·2H ₂ O	≥98.00%		
Boric acid	H ₃ BO ₃	≥99.00%		
Zinc sulfate heptahydrate	ZnSO ₄ ·7H ₂ O	≥99.00%		
Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	≥99.00%		
Sodium molybdate dihydrate	Na ₂ MoO ₄ ·2H ₂ O	≥99.00%		
Calcium chloride dihydrate	CaCl ₂ ·2H ₂ O	≥99.90%	Macklin Biochemical Technology Co., Ltd.	Shanghai, China
Manganese chloride tetrahydrate	MnCl ₂ ·4H ₂ O	≥99.00%		
Cobalt nitrate hexahydrate	Co(NO ₃) ₂ ·6H ₂ O	≥99.00%		
Sodium nitrate	NaNO ₃	≥99.00%	SHENTAI Chemical Industry Co., Ltd.	Tianjin, China
Potassium dichromate	K ₂ Cr ₂ O ₇	≥99.80%		

2.2. Preparation of Cr Source Medium and Cultivation of *Chlorella pyrenoidosa*

2.2.1. Preparation of Cr Source Medium

Chlorella pyrenoidosa was cultured in BG11 medium. Table 2 lists the compositions of BG11 medium used for this study. The BG11 medium has the following compositions (g/1 L ultrapure water): 1.5 NaNO₃; 0.04 K₂HPO₄; 0.75 MgSO₄·7H₂O; 0.006 C₆H₈O₇; 0.006 C₆H₁₁FeNO₇; 0.001 Na₂EDTA; 0.036 CaCl₂·2H₂O; 0.02 Na₂CO₃; 0.00286 H₃BO₃; 0.00182 MnCl₄·4H₂O; 0.00022 ZnSO₄·7H₂O; 0.0004 Na₂MoO₄·2H₂O; 0.00008 CuSO₄·5H₂O; 0.0005 Co(NO₃)₂·6H₂O.

Table 2. Compositions of BG11 medium used in study, (g/1 L ultrapure water).

Compositions	NaNO ₃	K ₂ HPO ₄	MgSO ₄ 7H ₂ O	C ₆ H ₈ O ₇	C ₆ H ₁₁ FeNO ₇	Na ₂ EDTA	CaCl ₂ 2H ₂ O	Na ₂ CO ₃	H ₃ BO ₃	MnCl ₄ 4H ₂ O	ZnSO ₄ 7H ₂ O	Na ₂ MoO ₄ 2H ₂ O	CuSO ₄ 5H ₂ O	Co(NO ₃) ₂ 6H ₂ O
Content	1.5	0.04	0.75	0.006	0.006	0.001	0.036	0.02	0.00286	0.00182	0.00022	0.0004	0.00008	0.0005

Weigh CrCl₃·6H₂O (molecular weight: 266.44) 0.51 g and dissolve with BG11, then transfer into 1000 mL volumetric bottle and set the volume to 1000 mL, then Cr(III) = 100 mg/L. BG11 was employed for diluting Cr(III) concentrations at 0.5, 1.0, 2.0 and 5.0 mg/L, respectively. The pH was regulated to a range of 6.8 to 7.1 using NaOH and HCl and was subsequently sterilized at 121 °C for 20 min. K₂Cr₂O₇ (molecular weight: 294.18) 0.28 g was weighed and diluted in constant volume according to Cr(III) configuration steps. After dilution, Cr(VI) concentrations were 0.5, 1.0, 2.0 and 5.0 mg/L, respectively.

2.2.2. Cultivation of *Chlorella pyrenoidosa*

Before inoculation, 1 mol/L of NaOH or HCl was used to adjust the pH of Cr source culture medium to 7.1 and then transferred to 1000 mL conical bottle. (DGL-50B, Shanghai Lichen Bangxi Instrument Technology Co., Ltd., Shanghai, China) at 121 °C for 20 min and cooled to 25 °C naturally. A total of 50 mL of *Chlorella pyrenoidosa* seed was inoculated into the sterilized medium (1000 mL). All the operations mentioned above were carried out on the clean bench (SW-CJ-1D, Shanghai Lichen Bangxi Instrument Technology Co., Ltd., China). The Cr source medium (1000 mL) was transferred to an illuminating incubator (GXZ-500B, Ningbo Jiangnan Instrument Factory, Ningbo, China) with a culture temperature of 25 °C, light intensity of 4000 Lux and light/darkness of 12/12. For experimental accuracy and to avoid microalgae sinking, three bottles were made for each group of experiments and were shaken three times per day.

2.3. Analysis of *Chlorella pyrenoidosa*

2.3.1. Analysis of *Chlorella pyrenoidosa* Lipid Content

We analyzed the biomass and lipid content of *Chlorella pyrenoidosa* after 25 days of being cultured in light. The algal liquid of 1000 mL was separated into batches and centrifuged at 8000 rpm for 15 min using a high-speed centrifuge. After removing the supernatant, the sediment was then transferred to a freeze dryer $-50\text{ }^{\circ}\text{C}$ until a constant weight was achieved [18]. The weight of the sample after it has dried is used to determine the biomass. Dried *Chlorella pyrenoidosa* powder (0.10–0.30 g) and quartz sand with a mass ratio of 1/1 were mixed and ground, sealed with filter paper (to prevent *Chlorella pyrenoidosa* powder from entering the distillation bottle during Soxhlet extraction) and transferred to a 250 mL Soxhlet extractor (SZF-06A, Shanghai Lichen Bangxi Instrument Technology Co., Ltd., China). Petroleum ether (200 mL) was added into a distillation flask equipped with a reflux condenser, heated to $90\text{ }^{\circ}\text{C}$ for 12 h and all solvents were removed at $75\text{ }^{\circ}\text{C}$. Lipid content of *Chlorella pyrenoidosa* (wt%) was calculated according to Equation (1).

$$\text{Lipid content of } Chlorella\ pyrenoidosa = (W_2/W_1) \times 100\%, \quad (1)$$

W_1 : mass of dried *Chlorella pyrenoidosa* powder, g; W_2 : mass of crude lipid, g.

2.3.2. Analysis of *Chlorella pyrenoidosa* Biomass

The absorbance of the cultured algal liquid was measured at 680 nm and the *Chlorella pyrenoidosa* biomass was determined according to the study of Lu et al. [16]. Equation (2) was used to calculate the biomass of *Chlorella pyrenoidosa*.

$$\text{Biomass} = 0.25 \times A_{680} + 0.001, (R^2 = 0.995) \quad (2)$$

A_{680} : absorbance value at 680 nm.

2.3.3. Analysis of *Chlorella pyrenoidosa* Enzyme Activity

Approximately 0.10 g *Chlorella pyrenoidosa* powder (fresh weight) was mixed with 1 mL extraction solution to be homogenized in an ice bath where they were centrifuged at $4\text{ }^{\circ}\text{C}$ for 10 min with the rate of $8000 \times g$ and then kept on ice. The pretreated sample was analyzed using a quantitative detection kit (cominbio, Suzhou, China) to measure the levels of reactive oxygen species (ROS), superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, peroxidase (POD) activity, acetyl-CoA carboxylase (ACC) activity, malic enzyme (ME) activity, phosphoenolpyruvate carboxylase (PEPC) activity, protein content and total sugar content.

2.3.4. Analysis of Cr(III) and Cr(VI)

GB7467-1987 was used to determine the concentration of Cr(VI), weigh 0.2829g of $\text{K}_2\text{Cr}_2\text{O}_7$ dried at $120\text{ }^{\circ}\text{C}$ for 2 h and prepare it into a Cr(VI) concentration of 100 mg/L solution and dilute it with deionized water to a Cr(VI) concentration of 1.0 mg/L solution. A total of 0.20 g of diphenylcarbazide was dissolved in 50 mL acetone and diluted with water to 100 mL to prepare the Cr(VI) color-developing agent. Cr (VI) solution (concentration of 0, 0.004, 0.01, 0.02, 0.04, 0.08, 0.12, 0.16 and 0.20 mg/L) were prepared by adding 0, 0.2, 0.5, 1, 2, 4, 6, 8 and 10 mL of Cr (VI) solution (concentration of 1.0 mg/L) into a 50 mL volumetric flask and adding ultrapure water to set the volume to 50 mL, then add 1 mL of H_3PO_4 solution (the volume ratio of H_3PO_4 to water is 1/1) and 2 mL of color developer respectively and shake well. The absorbance of the solution after color development was measured at 540 nm wavelength 5–10 min after color development. The absorbance of Cr(VI) at 540 nm is depicted in Figure 1. Equation (3) is for calculating the concentration of Cr(VI).

$$C = 1.8009 \times A_{540} - 0.0002, (R^2 = 0.9991) \quad (3)$$

C: concentration of Cr(VI), mg/L; A_{540} : absorbance value at 540 nm.

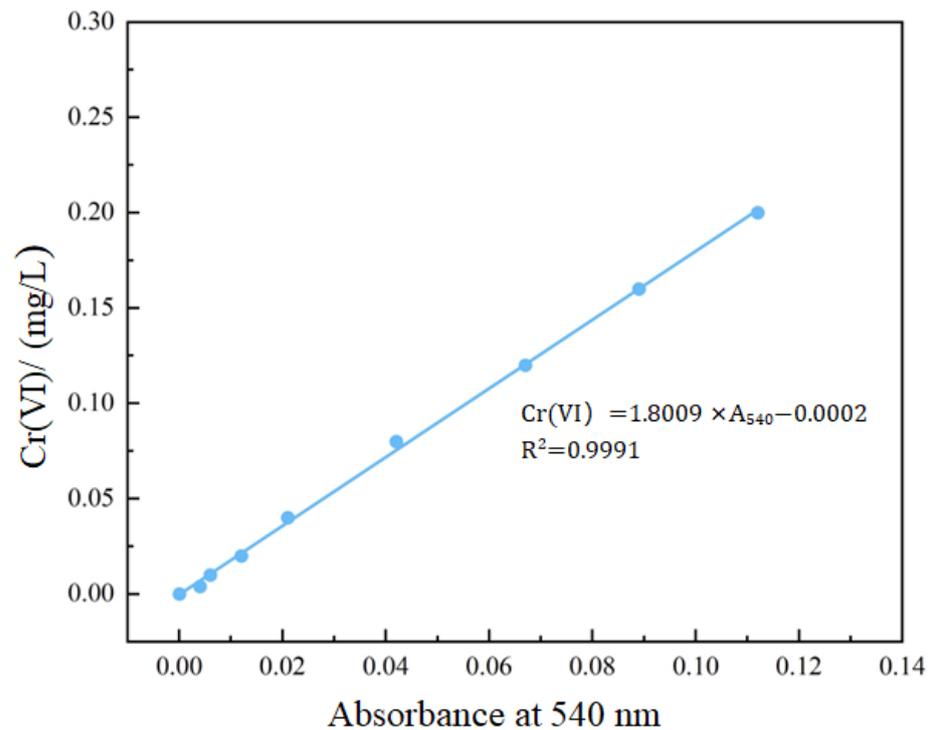


Figure 1. Absorbance of Cr(VI) at 540 nm.

The total chromium content was determined by the ICP-OES instrument and the content of Cr(VI) was subtracted from the total chromium content to obtain the content of Cr(III) in the liquid phase.

2.3.5. Statistical Analysis

The obtained biological data were subjected to statistical analysis using SPSS version 22.0. The statistically significant differences in the mean of the applied rates were analyzed with ANOVA followed by Duncan's test at a level of $p < 0.05$.

3. Results

Chlorella pyrenoidosa was cultivated in media containing Cr(III) and Cr(VI) at concentrations of 0.0, 0.5, 1.0, 2.0 and 5.0 mg/L. The biomass, total sugar, protein and lipid contents of *Chlorella pyrenoidosa* were then quantified. The impact of Cr(III) and Cr(VI) on lipid accumulation in *Chlorella pyrenoidosa* is presented in Figures 2 and 3 to provide clarity. The levels of SOD, MDA, POD, GSH and AsA were assessed following a 10 day culture of *Chlorella pyrenoidosa* under a controlled environment. The study elucidated the impact of Cr(III) and Cr(VI) on the antioxidant system and lipid peroxidation performance of *Chlorella pyrenoidosa*, with the findings featured in Figure 4. The removal ability of *Chlorella pyrenoidosa* to total Cr and the determination results of Cr(III) and Cr(VI) distribution in algal cells are shown in Figure 5. To uncover the regulatory mechanism of Cr(III) and Cr(VI) on the lipid accumulation of *Chlorella pyrenoidosa*, we investigated the alterations in acetyl-CoA carboxylase (ACC), malic enzyme (ME) and phosphoenolpyruvate carboxylase (PEPC) content after 10 days of *Chlorella pyrenoidosa* culture in a specific environment (Figures 6 and 7).

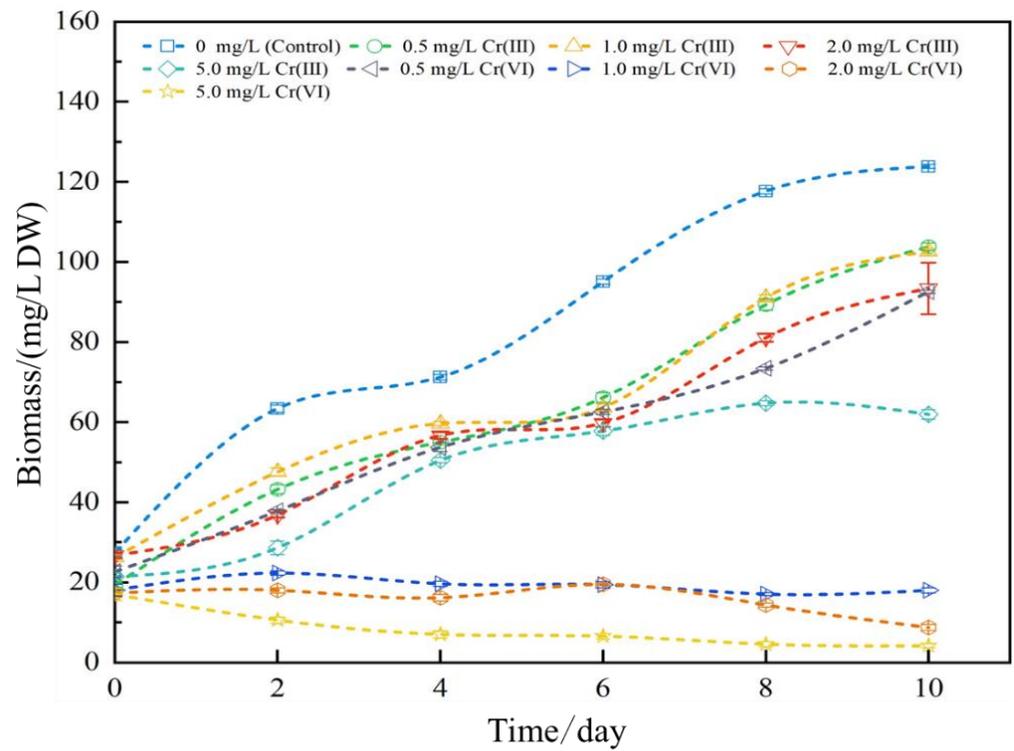


Figure 2. Effects of Cr(III) and Cr(VI) on biomass of *Chlorella pyrenoidosa*.

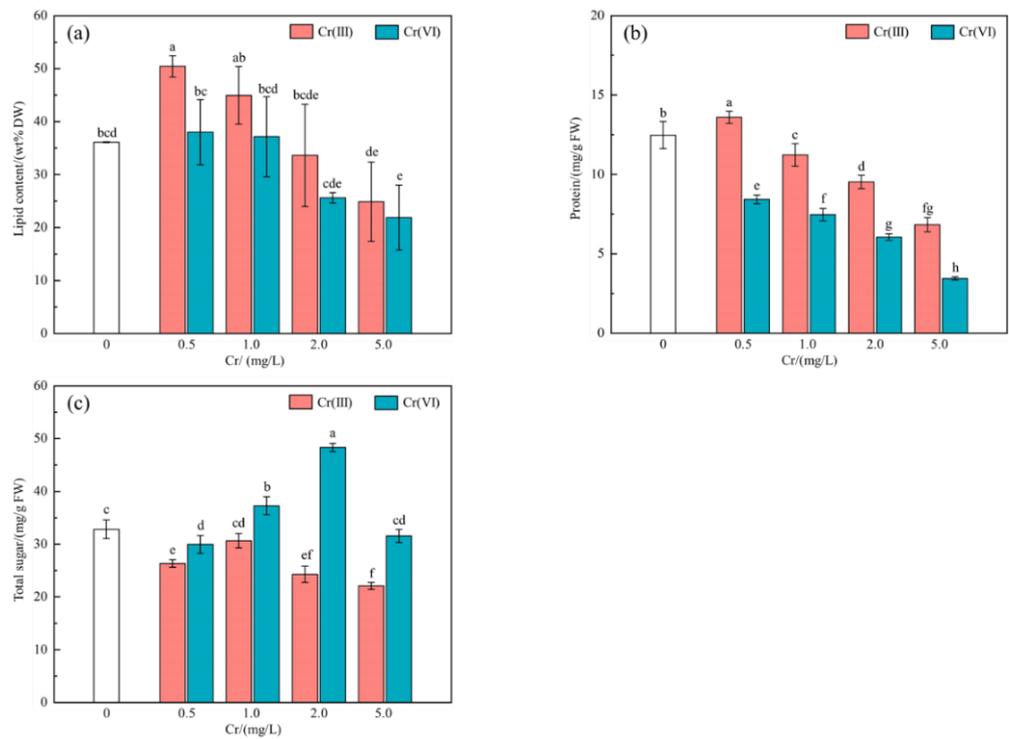


Figure 3. Effects of Cr(III) and Cr(VI) on (a) lipid content, (b) protein content and (c) total sugar content of *Chlorella pyrenoidosa*. The different letters are used to indicate the difference results after significant difference analysis ($p < 0.05$).

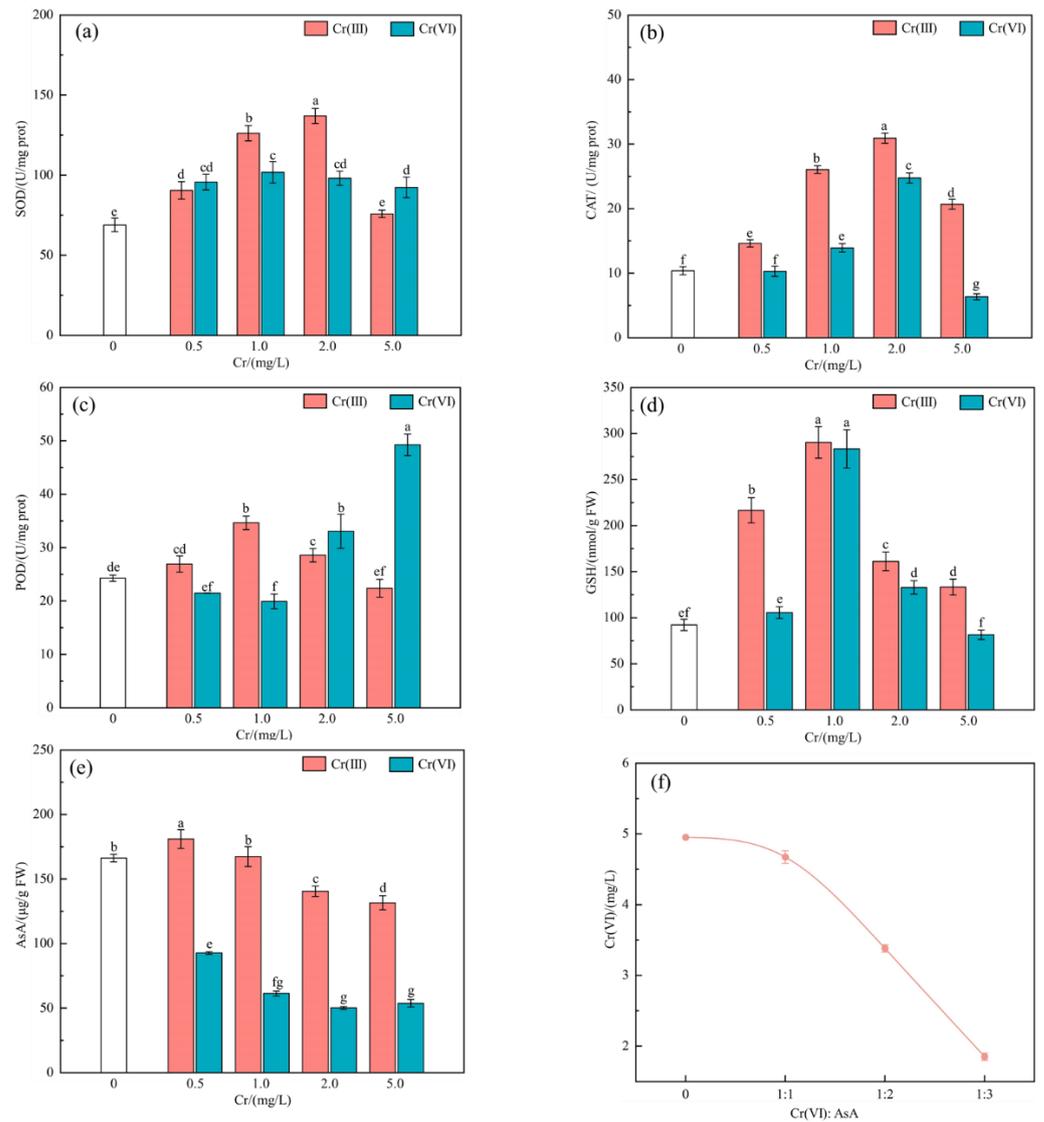


Figure 4. Response of *Chlorella pyrenoidosa* to Cr(III) and Cr(VI) stress and effect of AsA on Cr(VI) reduction rate. (a) SOD, (b) CAT, (c) POD, (d) GSH, (e) ASA and (f) Cr(VI) reduction rate. The different letters are used to indicate the difference results after significant difference analysis ($p < 0.05$).

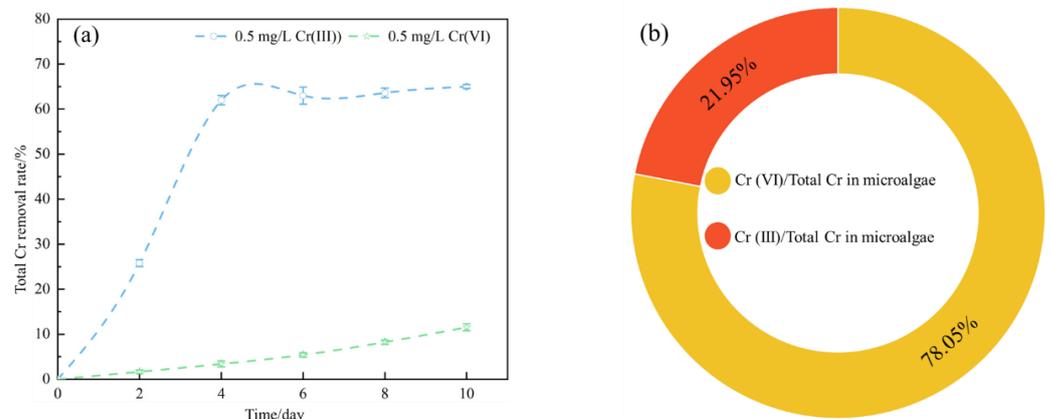


Figure 5. Removal of (a) total Cr and distribution of (b) Cr(III) and Cr(VI) contents in algal cells.

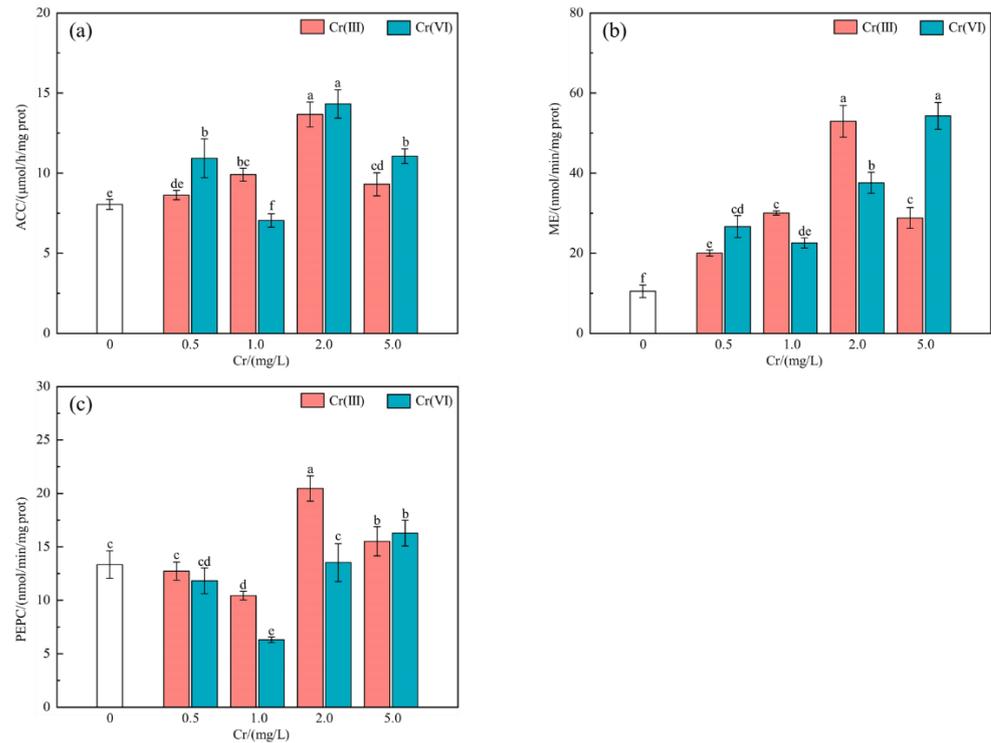


Figure 6. Effects of Cr(III) and Cr(VI) on (a) ACC, (b) ME and (c) PEPC activities. The different letters are used to indicate the difference results after significant difference analysis ($p < 0.05$).

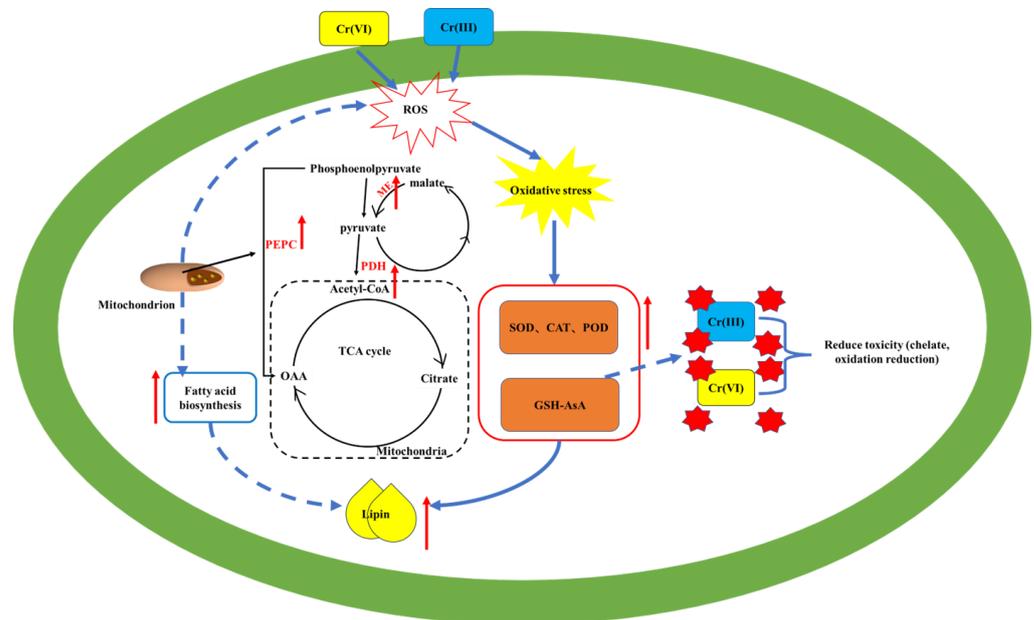


Figure 7. Mechanism of Cr(III) and Cr(VI) on lipid synthesis of *Chlorella pyrenoidosa*.

3.1. Effects of Cr(III) and Cr(VI) on Biomass

As illustrated in Figure 2, when the concentration of Cr(III) in the culture environment increased from 0.5 mg/L to 5.0 mg/L, the biomass of algal cells decreased from 104 mg/L to 62 mg/L, which was lower than the control group (124 mg/L). Similar results were found in the culture environment with the addition of Cr(VI), where the concentration of Cr(VI) increased from 0.5 mg/L to 2.0 mg/L, the biomass of *Chlorella pyrenoidosa* decreased from 93 mg/L to 9 mg/L and the growth of *Chlorella pyrenoidosa* was completely inhibited.

3.2. Cr(III) and Cr(VI) on Effect of Lipid, Protein and Total Sugar Content

Lipid content: when the concentration of both Cr(III) and Cr(VI) in the culture environment was 0.5 mg/L, the lipid content of *Chlorella pyrenoidosa* reached its maximum at 50.43 wt% and 38.00 wt%, respectively, compared to the control group (36.87 wt%) (Figure 3a). With the increase of Cr(III) or Cr(VI) concentration, lipid content tended to decrease (the lipid content corresponding to 5 mg/L Cr(III) decreased to 24.88 wt%; lipid content corresponding to 5 mg/L Cr(VI) decreased to 21.87 wt%).

Protein content: a low concentration of Cr(III) is helpful for the protein generation of *Chlorella pyrenoidosa* (Figure 3b). When Cr(III) is 0.5 mg/L, the protein content of *Chlorella pyrenoidosa* is 13.60 mg/g fresh weight (FW), 1.09 times that of the control group. As the concentration of Cr(III) in the medium continued to rise, there was a decrease in protein content within the algal cells. At a concentration of 1.0 mg/L FW, the protein content reached 11.23 mg/g FW, which was lower than the control group. However, the presence of Cr(VI) was unfavorable to the protein synthesis of *Chlorella pyrenoidosa*. The medium experienced a rise in Cr(VI) concentration from 0.5 to 5.0 mg/L, coinciding with a gradual decline in protein content from 8.42 mg/g FW to 3.45 mg/g FW.

Total sugar content: the total sugar content in *Chlorella pyrenoidosa* demonstrated an increasing trend followed by a decreasing trend as the concentrations of Cr(III) and Cr(VI) increased (Figure 3c). During the culture process with Cr(III) added, the total sugar content of *Chlorella pyrenoidosa* was lower than that of the control group at 32.83 mg/g FW and the highest value was achieved at 1.0 mg/L Cr(III), where the total sugar content of *Chlorella pyrenoidosa* was 30.66 mg/g FW. When 1.0 mg/L Cr(VI) was added to the medium, the total sugar content of *Chlorella pyrenoidosa* was 37.28 mg/g FW, higher than that of the control group. It reached the maximum value of 48.32 mg/g FW when Cr(VI) concentration was 2.0 mg/L.

3.3. *Chlorella pyrenoidosa* of Cr(III) and Cr(VI) Stress Response

After 10 days, *Chlorella pyrenoidosa* was cultured in a medium containing concentrations of 0.5–5.0 mg/L of Cr(III) and Cr(VI) sources. The levels of SOD, CAT, POD as well as non-enzymatic antioxidants (GSH and AsA) were measured in the algae cells to clarify the effects of Cr(III) and Cr(VI) stress on antioxidant and non-antioxidant enzymes of *Chlorella pyrenoidosa*.

Antioxidant enzymes (SOD, CAT and POD): the SOD and CAT activities of *Chlorella pyrenoidosa* in the medium supplemented with 0.5–5.0 mg/L Cr(III) exhibited a pattern of initially increasing and subsequently decreasing (Figure 4a,b). In the medium supplemented with 2.0 mg/L Cr(III), the SOD and CAT activities of *Chlorella pyrenoidosa* showed a trend of decreasing. SOD and CAT activities reached the maximum 136.98 U/mg prot and 30.91 U/mg prot respectively, which were 1.99 and 2.98 times that of the control group. A comparable trend was observed in the medium containing 0.5–5.0 mg/L of Cr(VI) as well. When the concentration of Cr(VI) was 1.0 mg/L and 2.0 mg/L, the activities of SOD and CAT reached the maximum of 101.81 and 24.76 U/mg prot, which were 1.48 and 2.39 times that of the control group. In Figure 4c, POD activity reached its maximum value at 1.0 mg/L Cr(III) and 5.0 mg/L Cr(VI), 34.65 and 49.24 U/mg prot, respectively, 1.43 and 2.03 times that of the control group (POD activity of the control group: 24.26 U/mg prot).

Non-enzymatic antioxidants (GSH and AsA): when Cr(III) and Cr(VI) were added to the culture environment increased from 0.5 mg/L to 1.0 mg/L, the GSH content of *Chlorella pyrenoidosa* under Cr(III) treatment increased from 216.64 nmol/g in FW (Cr(VI) environment (Figure 4d). The amount of 105.54 nmol/g FW was increased to the maximum value of 290.29 nmol/g FW (GSH content in Cr(VI) environment (283.33 nmol/g FW) in the control group (GSH content: 3.15 and 3.07 times of 92.19 nmol/g FW), Cr(III) and Cr(VI) concentrations continued to increase and GSH content began to decline. The AsA content of *Chlorella pyrenoidosa* decreased with the increase of Cr(III) and Cr(VI) concentrations in the culture environment (Figure 4e). Compared with the control group, the AsA content was higher only under 0.5 and 1.0 mg/L Cr(III) conditions. To further understand the

influence of AsA on Cr(VI) in *Chlorella pyrenoidosa*, the reduction rate of Cr(VI) at different ratios of Cr(VI)/AsA was explored (Figure 4f). With the increase of ascorbic acid, the reduction rate of Cr(VI) gradually increased. In water containing 5.0 mg/L Cr(VI), the three concentration ratios of Cr(VI)/AsA were 1/1, 1/2 and 1/3, respectively, and the reduction rates were 5.62%, 27.62% and 45.27%.

3.4. Distribution of Cr(III) and Cr(VI) in Algal Cells

A concentration of 0.5 mg/L for both Cr(III) and Cr(VI) was chosen as the specific culture conditions to assess the capability of *Chlorella pyrenoidosa* in removing total Cr from the culture environment. Figure 5a illustrates that the removal rate for Cr(III) reached 65.00% by the end of the culture period. For Cr(VI), there was no significant decrease in total Cr concentration during cultivation, and the final removal rate was only 11.53%.

The distribution of Cr(III) and Cr(VI) within algae cells was analyzed by selecting a Cr(VI) concentration of 0.5 mg/L as a specific culture condition. According to Figure 5b, the distribution of Cr(VI) and Cr(III) content in algae cells was observed under specific culture conditions with 0.5 mg/L of Cr(VI). The active compounds found in microalgae transform Cr(VI) into Cr(III) partially, thus reducing the toxicity of Cr(VI).

3.5. The Effect of Cr (III) and Cr (VI) on the Activity of ACC, ME and PEPC

Chlorella pyrenoidosa was cultured in Cr(III) and Cr(VI) source medium with concentrations of 0.5–5.0 mg/L. ACC, ME and PEPC were found in algal cells after a period of 10 days. The objective is to investigate the impact of Cr(III) and Cr(VI) stress on the enzymes involved in lipid synthesis in *Chlorella pyrenoidosa*.

Acetyl-CoA carboxylase (ACC): the ACC activity of *Chlorella pyrenoidosa* varied with the concentration of Cr (Figure 6a). Compared to the control group (8.05 $\mu\text{mol/h/mg prot}$), it initially increased and then decreased. ACC activity in Cr(III) and Cr(VI) at 2.0 mg/L reached a maximum of 13.67 and 14.32 $\mu\text{mol/h/mg prot}$, respectively, which was 1.70 and 1.78 times that of the control group. The results were inconsistent with the results of the lipid content of *Chlorella pyrenoidosa* (when Cr(III) or Cr(VI) concentrations were both 0.5 mg/L in the culture environment, the lipid content of *Chlorella pyrenoidosa* reached the maximum 50.43 wt% and 38.00 wt%).

Malic enzyme (ME): the activity of ME was higher than the control group in the concentration range of 0.5–5.0 mg/L for Cr(III) and Cr(VI) (Figure 6b). At 2.0 mg/L Cr(III) and 5.0 mg/L Cr(VI), the maximum prot values were 52.96 and 54.31 nmol/h/mg, respectively, which were 5.03 and 5.16 times that of the control group.

Phosphoenolpyruvate carboxylase (PEPC): at concentrations of 0.5 and 1.0 mg/L Cr(III), the PEPC activity of *Chlorella pyrenoidosa* was 12.72 nmol/min/mg prot and 10.43 nmol/min/mg prot, respectively, lower than that of the control group (Figure 6c). At 0.5 mg/L and 1.0 mg/L Cr(VI) concentrations, the PEPC activity of *Chlorella pyrenoidosa* was 11.82 nmol/min/mg prot and 6.31 nmol/min/mg prot, respectively, lower than that of the control group.

4. Discussion

4.1. Effects of Cr(III) and Cr(VI) on Biomass

Cr(VI) can be converted into Cr(V) complexes by physiological reducing agents, including NADPH, various pentoses, glutathione and others. The toxic effects of Cr are associated with the reduction of Cr(VI) to a lower oxidation state, particularly the instantaneous formation of Cr(V). The Cr(V) complex reacts with H_2O_2 to produce a significant amount of OH^- radicals instead of the corresponding O^{2-} , which can directly induce alterations in DNA and the synthesis of other harmful compounds [19]. Therefore, it is particularly important to explore the effects of Cr(III) and Cr(VI) on the physiological and biochemical indexes of *Chlorella pyrenoidosa*. It has been found that when the concentration of Cr(VI) increases from 5 ppm to 10 ppm, the growth rate of microalgae will be adversely affected, resulting in a decrease in biomass density [17]. Cr(VI) induces oxidative stress in

microalgal cells, inhibiting electron transport and the absorption, transfer and utilization of light energy, resulting in this phenomenon [20].

4.2. Cr(III) and Cr(VI) on Effect of Lipid, Protein and Total Sugar Content

Lipid content: microalgae synthesize lipids by using CO₂ or glucose under environmental stress (Figure 3a) [21]. Cr(III) and Cr(VI) as stress factors had positive effects on lipid accumulation in microalgae in the culture environment. Obviously, the addition of Cr(III) or Cr(VI) during culture triggered the production of a large number of ROS by microalgal cells, which activated different protective mechanisms and intensified the accumulation of secondary metabolites (such as lipids) [22]. According to the report, it was found that *Scenedesmus quadricauda* increased the lipid content of Cr(VI) at low concentrations [20]. However, high concentrations of Cr can cause damage to intracellular structures and inhibit cell growth, thereby reducing lipid content.

Protein content: Sharma et al. also reported similar results, diluting concentrations of 75% wastewater to cultivate algae group MAC2 (*Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus dimorphus*, *Kirchnella* and *Microcoleus*) (Figure 3b). After 10 days, the protein content was higher than that of the control group, and when the wastewater concentration was increased to 100%, the protein content was lower than that of the control group [23]. The increase in protein content may result from the synthesis of certain stress proteins or metal-chelating proteins. The presence of high concentrations of Cr(III) and Cr(VI) will lead to protein degradation and is used for the growth of *Chlorella pyrenoidosa* under high Cr stress [24].

Total sugar content: the increase in total sugar content can be attributed to certain changes in the photosynthetic mechanism, enhanced cell division and synthesis of proteins and osmoprotectants (Figure 3c) [25]. It has been shown that the accumulation of total sugars in microalgae can be enhanced under heavy metal stress [26]. Kafil et al. reported that microalgae accumulate total sugars under heavy metal stress, which is a mechanism to combat heavy metal stress [17]. The stress mechanism may be that microalgae produce total sugars in the heavy metal environment and the newly formed total sugars bind to heavy metals in the culture environment on the cell wall of microalgae to reduce the damage of heavy metals to microalgae. The possible reason for the decrease in total sugar is that the degradation of total sugar caused by high concentrations of Cr(III) and Cr(VI) provides nutrients for algal cell growth under high Cr stress.

4.3. *Chlorella pyrenoidosa* of Cr(III) and Cr(VI) Stress Response

Antioxidant enzymes (SOD, CAT and POD): some external environmental factors of stress such as heavy metals, organic acid and salt, can induce algal cells to form the ROS (Figure 4). Intracellular ROS such as hydroxyl radical (OH⁻), hydrogen peroxide (H₂O₂), peroxide(O²⁻) and singlet oxygen (1O₂) have strong oxidation potential and can cause fatal damage to algal cells [27]. SOD can convert O²⁻ to H₂O₂ and O₂, while CAT and POD can convert H₂O₂ to O₂ and H₂O. SOD, CAT and POD are frequently utilized as biomarkers to measure cell response to external stress [28]. Therefore, this chapter focuses on the changes in SOD, CAT and POD contents in *Chlorella pyrenoidosa* cells under Cr(III) and Cr(VI) stress. Relevant studies have confirmed that a low concentration of Cr(VI) will increase the SOD and CAT activities of *C. vulgaris*, while high concentration of Cr(VI) will decrease the SOD and CAT activities of microalgae [13]. It can be seen from Figure 4 that the addition of Cr(III) and Cr(VI) at different concentrations has significant effects on the antioxidant activity of *Chlorella pyrenoidosa*. The reason for these results is that when microalgae cells are exposed to low concentrations of Cr, they protect themselves from excessive ROS by increasing the activity of antioxidant enzymes, thereby preventing or reducing oxidative damage. When Cr concentrations exceed the threshold, the antioxidant enzyme system cannot maintain a dynamic balance of free radical production and clearance and the intracellular antioxidant enzyme system produces certain oxidative damage, leading to a decrease in antioxidant capacity.

Non-enzymatic antioxidants (GSH and AsA): to mitigate the toxic effects of ROS, plant cells have developed different defense mechanisms. The initial reaction of oxidative stress is related to the change in the glutathione-ascorbate cycle and catalase activity, which is responsible for the degradation of H₂O₂ [29]. Branca et al. observed that in the GSH-Cr(VI) system, glutathione reacts with chromate to produce a thioester compound. This thioester substance can be efficiently reduced by free tripeptide molecules. In systems that contain chromates, sugars and glutathione, Cr(VI) interacts with a sugar ligand (or both sugar and GSH) to form an ester that can be easily reduced by GSH. The resulting Cr(V) ion is subsequently stabilized by coordination with the sugar ligand [30]. Therefore, this chapter focuses on the changes in GSH and AsA contents of *Chlorella pyrenoidosa* in Cr(III) and Cr(VI) medium with different concentrations. As shown in Figure 4d, relevant studies have also confirmed that the GSH content of microalgae cultured in the range of 0–40 mg/L Cr(VI) has similar changes [31]. Schiavon et al. reported that the increased GSH content may be related to the reduction of Cr(VI) to Cr(III), which is less toxic [32]. In this experiment, Cr(III) and Cr(VI) act as environmental stressors, causing a significant increase in the GSH (glutathione) content within algal cells. This increase helps to remove excess reactive oxygen species (ROS) from microalgae. However, when the Cr concentration increases to exceed the threshold of microalgae, it will cause damage to GSH, resulting in a decrease in GSH content. As shown in Figure 4e, during oxidative stress, AsA undergoes oxidation to form dehydroascorbic acid (DHA), resulting in a decrease in AsA content particularly under heavy metal stress. Kováčik et al. also found similar results, AsA was lower than the control group in the presence of Hg and Pb and AsA was protected from destruction of metal toxicity by regulating ROS/NO balance *C. subellipsoidea* [33]. To further understand the influence of AsA on Cr(VI) in *Chlorella pyrenoidosa*, the reduction rate of Cr(VI) at different ratios of Cr(VI)/AsA was explored (Figure 4f). The findings indicate that the increase in AsA usage is associated with higher rates of Cr(VI) reduction, as observed by Yen et al. who found that Cr(VI) reduction in microalgae may have two synergistic mechanisms: biological mechanism (enzyme pathway) and abiotic mechanism (GSH release) [34]. Yen et al. also observed the average reduction rate of Cr(VI) in microalgae by three treatment methods: live cells, dead cells and cell-broken super-natant (Table 3) [34].

Table 3. Average reduction rate of Cr(VI) by different treatment methods [34].

	Living Cell	Dead Cell	Cell Crushing Supernatant
Decrement rate (mg/L/day)	3.73	1.52	0.38
Specific reduction rate (mg/g/day)	2.07	0.95	-

Note: Performed at an initial concentration of 10 mg/L Cr(VI).

4.4. Distribution of Cr(III) and Cr(VI) in Algal Cells

In Figure 5a, The removal of Cr(III) is made possible by the binding between cell wall functional groups and metal ions. Moreno et al. discovered that Cr(III) was found when *Tetradismus* sp. and *Scenedesmus* sp. were treated together, and *Ascomycota* sp., microalgae exhibited irregular and porous cell walls. The interaction between the functional groups of the cell wall and Cr(III) changed the morphological characteristics of microalgae, enhancing the adsorption energy of Cr(III) [35]. For Cr(VI), Lu et al. pointed out that when the concentration of Cr(VI) exceeds a certain limit, the growth of *Chlorella vulgaris* is significantly inhibited, resulting in the inhibition of Cr removal [16].

According to Figure 5b, The active compounds found in microalgae transform Cr(VI) into Cr(III) partially, thus reducing the toxicity of Cr(VI). Shen et al. pointed out that the decrease in Cr(VI) toxicity is related to the growth media that induce microalgae growth (such as organic carbon, nitrogen and phosphorus) and different types of organic carbon sources can cause different distributions of Cr(VI) and Cr(III) content in microalgae cells [6]. Cr(VI) can react with plant chelating elements and ATP synthase to form hydrogen and ion bonds. Plant chelating elements are rich in cysteine, which can chelate metal ions through

thiol (-SH) to reduce the toxicity of Cr(VI). In addition, after entering the cell, Cr(VI) not only binds to macromolecules such as phytochelatin, metallothionein and chlorophyll, but also binds to enzymes in the cell, serving as an important detoxification mechanism [36]. The active substance converting Cr(VI) into Cr(III) in algae cells may be ascorbic acid (Figure 4e,f).

4.5. Effect of Cr (III) and Cr (VI) on Activity of ACC, ME and PEPC

In Figure 6a, compared with the control group, the ACC activity of *Chlorella pyrenoidosa* first increases and then decreases with the concentration of Cr, and is inconsistent results in lipid content of *Chlorella pyrenoidosa*, this is due to the fact that ACC activity is regulated not only at the transcriptional level, but also after transcription [37–39]. However, Cr induces an overproduction of reactive oxygen species, which leads to a decrease in conversion efficiency of light energy and causes damage to lipids, proteins and other macromolecules [40–42]. This ultimately results in a decrease in lipid content and an increase in ACC activity.

Ikaran et al. reported that under nitrogen stress, the ME gene of *Chlorella vulgaris* was significantly upregulated and the total lipid content was increased (Figure 6b) [37]. Ren et al. reported that adding malic acid promotes *Schizochytrium* sp. to enhance NADPH supply and increase lipid content [43]. Increased ME activity results in a notable rise in the concentration of malate and citrate within the cell, causing an increase in their outflow as well. This increase in concentration enhances the catalytic process of converting malate into pyruvate, which can then be transformed into ACC through the action of pyruvate dehydrogenase [44]. These results suggest that ME may be responsible for both, providing reducing capacities for fatty acid synthesis and ACC under Cr stress, promoting lipid accumulation.

Previous studies have shown that the decrease in PEPC activity is beneficial to fatty acid synthesis (Figure 6c) [45] which is corroborated by the lipid content in Figure 3a. This is because downregulation of the PEPC gene increases the availability of phosphoenolpyruvate and converts it into pyruvate, which is catalyzed by pyruvate dehydrogenase to generate ACC and promote lipid synthesis [37]. Therefore, the low concentration of Cr can promote the synthesis of lipids, the lipid content is 50.43 wt% and 44.95 wt% (0.5 mg/L and 1.0 mg/L Cr(III)), respectively, and Cr (VI) concentrations are 0.5 mg/L and 1.0 mg/L Cr(VI) with lipid content of 38.00 wt% and 37.15 wt%, respectively. The maximum value of PEPC activity in algal cells was achieved at concentrations of 2.0 mg/L Cr(III) and 5.0 mg/L Cr(VI), which were 1.53 and 1.22 times higher than the control group, respectively. This cannot be explained by the demand for carbon in the TCA cycle. Other studies have shown that *Chlamydomonas reinhardtii* increases PEPC when subjected to nitrogen stress [46,47]. This may be an active molecular mechanism by which algal cells respond to heavy metal stress, resulting in different PEPC activities in Cr(III) and Cr(VI).

4.6. Mechanism of Influence of Cr(III) and Cr(VI) on Lipid Synthesis of *Chlorella pyrenoidosa*

Based on the above results, we can speculate the mechanism of detoxification and lipid production of Cr(III) and Cr(VI) by *Chlorella pyrenoidosa* (Figure 7). Algal cells are stimulated by Cr(III) and Cr(VI) to produce reactive oxygen species. To mitigate cell damage from oxidative stress and stimulate immune responses, Cr(III) and Cr(VI) engage in a complex chelation reaction with Cr(III) and Cr(VI), promoting the upregulation of antioxidant enzymes (SOD, CAT and POD) as well as non-enzymatic antioxidants (GSH and AsA). Lipid synthesis is stimulated while the TCA cycle in mitochondria is activated to produce secondary metabolites (such as ACC, ME and PEPC) that promote lipid synthesis.

5. Conclusions

Chlorella pyrenoidosa was cultured with Cr(III) and Cr(VI) at 0–5.0 mg/L. The analysis focused on the impact of Cr(III) and Cr(VI) on the lipid content, biomass, protein, total sugars, non-enzymatic antioxidants and enzyme activities (SOD, CAT, POD, ACC, ME and

PEPC) of *Chlorella pyrenoidosa*. The growth and lipid synthesis of *Chlorella pyrenoidosa* were examined in response to Cr(III) and Cr(VI). The following are the main conclusions.

- (1) When Cr(VI) concentration was in the range of 1.0–2.0 mg/L, it was beneficial to accumulate total sugar (37.28–48.33 mg/g FW > 32.83 mg/g FW in the control group). Concentrations of Cr(III) and Cr(VI) ranging from 0.5 to 1.0 mg/L were found to be conducive to lipid accumulation, with a weight percentage of 37.15–50.43 wt%, compared to the control group with 36.87 wt%. At a concentration of 0.5 mg/L of Cr(III), protein accumulation was observed to be higher (13.60 mg/g FW) compared to the control group (12.47 mg/g FW), indicating a beneficial effect.
- (2) Cr(VI) is 100 times more toxic than Cr(III), is easy to dissolve and has a strong oxidation capacity, changing the expression of genes and proteins. Microalgae cope with Cr(VI) stress by regulating the activity of antioxidant enzymes, such as increasing the activities of SOD, CAT and POD. Additionally, algal cells actively convert the more toxic Cr(VI) to Cr(III) through substances like GSH and AsA.
- (3) Cr(III) and Cr(VI) can help *Chlorella pyrenoidosa* improve the key enzyme of lipid synthesis in microalgae, but only under the conditions of 0.5–1.0 mg/L Cr(III) and Cr(VI), the lipid level of *Chlorella pyrenoidosa* is higher than that of the control group. Although the high Cr environment can help the key enzymes of lipid synthesis in microalgae maintain high activity to produce more lipids, the excessive production of reactive oxygen species induced by high Cr will also accelerate the destruction of the synthesized lipids and other macromolecules, which is unfavorable to the final result.

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