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Bioremoval efficiency and metabolomic profiles of cellular responses of *Chlorella pyrenoidosa* to phenol and 4-fluorophenol

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ABSTRACT

We examined the growth tolerance, bioremoval efficacy, and metabolomic profiles of the cellular responses of *Chlorella pyrenoidosa* to phenol and 4-fluorophenol. We found that *C. pyrenoidosa* can tolerate exposure to 100 mg/L of phenol and 4-fluorophenol, and the growth of algal cells had a significant hormesis of inhibition first and then promotion. Up to 70% bioremoval of phenol and 4-fluorophenol may occur after 240 h of treatment. Superoxide dismutase (SOD) and catalase (CAT) activities, malondialdehyde (MDA) content, and reactive oxygen species (ROS) in algal cells in the phenol- and 4-fluorophenol-treated groups were similar or lower than in the control group. Furthermore, photosynthetic pigment and glycerophospholipid contents were significantly upregulated in both phenol- and 4-fluorophenol-treated groups, as indicated by the metabolomic analysis of *C. pyrenoidosa*, resulting in the vigorous growth of algal cells compared to the control group. Therefore, *C. pyrenoidosa* can be an excellent biosorbent for phenol and 4-fluorophenol.

Key words: *Chlorella pyrenoidosa*, 4-fluorophenol, metabolomics, phenol, removal

HIGHLIGHTS

- *Chlorella pyrenoidosa* can well tolerate 100 mg/L of phenol or 4-fluorophenol exposure.
- 100 mg/L phenol or 4-fluorophenol can be bioremoved by >70% after 240 h of treatment.
- Significant hormesis in algal growth was observed during the treatment.
- Growth promotion is attributed to chlorophyll and glycerophospholipid accumulation.

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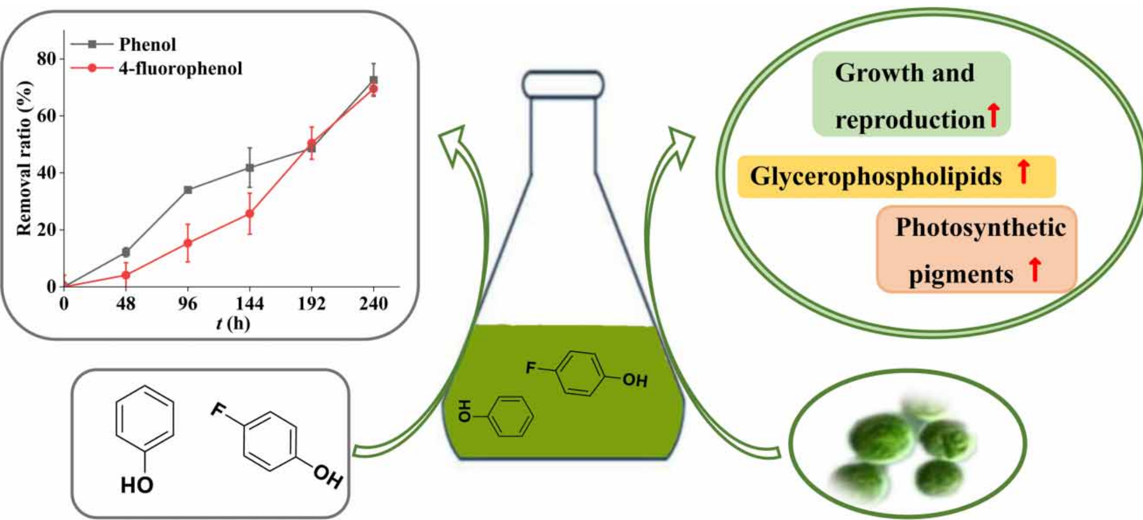
GRAPHICAL ABSTRACT



1. INTRODUCTION

Phenol and halophenols are widely used to produce pharmaceuticals, herbicides, dyes, disinfectants, and preservatives (Brycht *et al*. 2016; Fawzy & Alharthic 2021). Environmental pollution involving these compounds is directly proportional to extensive industrialization. Such substances have been detected in various water bodies with a broad range of concentrations (Wang 2022). For instance, the concentration of 4-chlorophenol reached 37.60–47.81 ng/L in the Yangtze River of China (Zhu *et al.* 2022). In contrast, it varies between 100 and 1,000 mg/L in effluents from relevant industries (Patel *et al.* 2022). Phenol concentration in wastewater varies between 10 and 300 mg/L but reaches 4.5 g/L in highly polluted ones (Al-Khalid & El-Naas 2012). Phenol and halophenols, especially halophenols with one or more halogen atoms, are resistant to degradation, persisting in the environment, and resulting in a range of environmental pollution and human health risks (Ge *et al.* 2017; Juksu *et al.* 2019). Halophenols have been categorized as priority pollutants in Europe, the USA, and China, given their hazardous nature (Duan *et al.* 2019). Consequently, the elimination of phenol and halophenol is an important requirement for eco-environmental safety.

Various physicochemical and biological processes have been successfully explored to remove phenol and halophenols. Biological treatment is eco-friendly due to its minimal risk of toxic secondary products compared with physicochemical techniques. Microalgae-mediated bioremediation is highly preferred as a candidate for pollutant removal (Ansar *et al.* 2022; Couto *et al.* 2022). Microalgae are nontargets for antibiotics (Escudero *et al.* 2020; Sawaya *et al.* 2022). Microalgae can be reused as fuel, pigments, fertilizers, and medicine according to the pollutant removal mechanism after proper treatments (Andreotti *et al.* 2020; Jalilian *et al.* 2020; Ong *et al.* 2021; Reddy *et al.* 2021). However, phenol and halophenol treatment by algae has been studied far less than that of fungi and bacteria (Fawzy & Alharthic 2021).

Biosorption, bioaccumulation, and biodegradation are essential functions played by microalgae while removing organic pollutants (Xiong *et al.* 2016; Chen *et al.* 2021a). Biosorption is the first step in the bioaccumulation process, where pollutants are absorbed into cells by metabolism-dependent active transport systems (Novák *et al.* 2020). The interaction between algal cells and pollutants is complex, as efficient pollutant removal is affected by potential adverse effects on algal cells. Biosorption is challenging when pollutant concentrations are high. In such cases, photosynthesis decreases and lipid peroxidation is produced as a result of pollution stress caused by pollutants, leading algae cells to irreversible injuries or death (Chin *et al.* 2019; Chen *et al.* 2021b; Fawzy & Alharthic 2021; Zhai *et al.* 2022). Therefore, it is necessary to analyse the growth and physiological properties of algal cells when exposed to pollutants and their removal efficacy.

Algal-based pollutant removal usually depends on the substance structure and the algal species (Xiao *et al.* 2021). *Chlorella pyrenoidosa* is one of the most representative green algae and is associated with strong adaptability, rapid reproduction, and sensitivity to pollutants. Phenol and 4-fluororphenol are widely used. Consequently, the availability of these substances increases in the environment. Phenol and 4-fluororphenol differ in structure, toxicity, and biodegradability. In this study,

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the removal efficacy of phenol and 4-fluororphenol by *C. pyrenoidosa* was studied. The effect of phenol and 4-fluorophenol on the growth rate, antioxidant enzyme activity, and metabonomics of *C. pyrenoidosa* was explored. The results are beneficial for examining the remediation potential and mechanism of phenol/4-fluorophenol wastewater by microalgae.

2. MATERIALS AND METHODS

2.1. *C. pyrenoidosa* growth inhibition test

C. pyrenoidosa (collection no. FACHB-5) was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. Algal cells were grown in a 250-mL flask containing 100 mL of BG11 medium. All flasks were kept in an artificial climate box with a 25 ± 0.2 °C, 12:12 h light (4,000 lux)-dark cycle. All flasks were shaken manually three times a day.

Phenol and 4-fluorophenol were dissolved in methanol and the volume of methanol in the exposure solution was always $\leq$ 0.5%. Solutions of 0.5% and non-methanol were set as the solvents and blank controls. A *t*-test was conducted between those two controls and $p > 0.05$ was obtained, indicating that methanol toxicity could be negligible under this threshold. The initial algal density was adjusted to (1–2) $\times 10^5$ cells/L in the exposure solutions. The initial concentrations of phenol and 4-fluorophenol were 0, 10, 50, and 100 mg/L. An algal density (ρ, cells/mL) measurement was conducted by haemocytometer every 24 h until 240 h and the inhibition ratio (%) = $(\rho_{control} - \rho_{treatment})/\rho_{control}$ was calculated (OECD 2006).

2.2. Determination of phenol and 4-fluorophenol

The phenol and 4-fluorophenol concentrations in the exposure solution were determined using high-performance liquid chromatography (HPLC, Agilent 1200, ZORBAX RX-C18) (4.6 mm × 150 mm, 5 μm) with a diode array detector (testing λ was 270 nm). The removal ratio (%) of phenol/4-fluorophenol was calculated according to the ratio of its removal concentration to its initial concentration. Additionally, molecular spectrum scanning (λ from 250 to 400 nm) of phenol/4-fluorophenol cultured in the BG11 medium was conducted every 24 h until 240 h to examine the abiotic degradation of phenol/4-fluorophenol.

2.3. Determination of physiological and biochemical indexes

Ten mL of algal solution was centrifuged at 4,000 rpm for 10 min. Next, the algal pellet was suspended in 10 mL phosphate buffer saline (PBS, 0.01 mol/L, pH 7.8). Subsequently, the solution was ultrasonicated on ice for 15 min at 200 W, ultrasonic time: 3 s, and rest time: 7 s to break cells. Later, the crude enzyme solution was obtained by centrifugation at 4,000 rpm. The total soluble protein (TSP), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and reactive oxygen species (ROS) contents were measured using a commercial kit (Keming, Suzhou, China).

2.4. Metabolomics

Algal cells treated with 100 mg/L phenol/4-fluorophenol for 96 h were collected for metabolomic analysis. Briefly, 20 mL of samples were ultrasound-crushed, freeze-dried, and ultrasonically extracted with 10 mL of methanol. The extracted liquid was centrifuged (10,000 g for 10 min), filtered (0.2 μm filter membrane) and used for metabolite detection. An Agilent 6546 UPLC-Q/TOF-MS containing a ZORBAX Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm) was used to run the samples. The mobile phases were (A) H_2O with 0.1% ammonium acetate and (B) methanol. Gradient elution was performed at a flow rate of 0.4 mL/min under the following programme: held at 10% B from 0 to 3 min; then, B changed linearly from 10 to 70% in the next 3–15 min. Over the next 0.10 min, B changed to 100% and was held for 3 min. The column temperature was kept at 30 ° C. The MS injection volume for each sample was 2 μL. The operating parameters for the Q/TOF-MS in both positive and negative ion modes were: gas temperature 325 °C, drying gas flow 10 L/min, nebulizer pressure 40 psi, mass scan range from 50 to 1,500 m/z, and acquisition rate of 200 ms/spectrum.

The raw data were converted into CEF format using mass hunter profinder software (Agilent Corp., 10.1) and imported into Mass Profiler Professional software (version 15.1) for further analysis. A one-way analysis of variance (ANOVA) was used to determine the differences among the control, phenol, and 4-fluorophenol-treated groups. A *t*-test was used to determine the differences between the control vs. phenol-treated groups and the control vs. 4-fluorophenol-treated groups. A significant difference occurred when $p < 0.05$. Unsupervised principal component analysis (PCA) and orthogonal partial least

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squares-discriminant analysis (OPLS-DA) were applied to obtain an overview of the chemical variations among treatments. METLIN PCDL B.08.00 was used to identify the compounds. The differential metabolites were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the corresponding metabolic pathways were searched.

3. RESULTS AND DISCUSSION

3.1. The tolerance and removal efficacy of *C. pyrenoidosa* to phenol and 4-fluorophenol

The tolerance of *C. pyrenoidosa* to phenol and 4-fluorophenol exposure was evaluated based on a growth inhibition test. The results are shown in Figure 1(a) and 1(b). The tolerance was significantly correlated with exposure time and concentration. Each 10 mg/L of phenol and 4-fluorophenol had a weak promoting effect on the growth of algal cells, while the first inhibited and then promoted growth trend was observed in the culture of each 100 mg/L of phenol and 4-fluorophenol. The most significant promotional effect of phenol and 4-fluorophenol on the growth of algal cells was observed at 96 h. The above results can be summarized as the U-shaped effect found in many microalgae after being stimulated by exogenous substances. Microalgae can maintain the vitality and stability of the algal system by increasing the number of algal cells after stimulation by microplastics (Li *et al*. 2022). Namely, the increase in algal density could be an apparent stress-resistance response of the microalgae.

Phenol and 4-fluorophenol had good stability in the BG11 medium in the 240 h tested (Supplementary material, Figure S1). However, within the algal cells, it was found that 10 mg/L phenol could be removed in 144 h. In contrast, the removal ratios of 50 and 100 mg/L phenol after 240 h of exposure were 85.23 and 72.64%, respectively (Figure 1(c)). The removal efficiency of 4-fluorophenol by algal cells was slightly lower than that of phenol, with removal ratios of 10, 50, and 100 mg/L 4-fluorophenol after 240 h of exposure of 91.92, 80.46, and 70.51%, respectively (Figure 1(d)). Compared with phenol, the tolerance and removal efficacy of 4-fluorophenol by *C. pyrenoidosa* decreased slightly in this study.

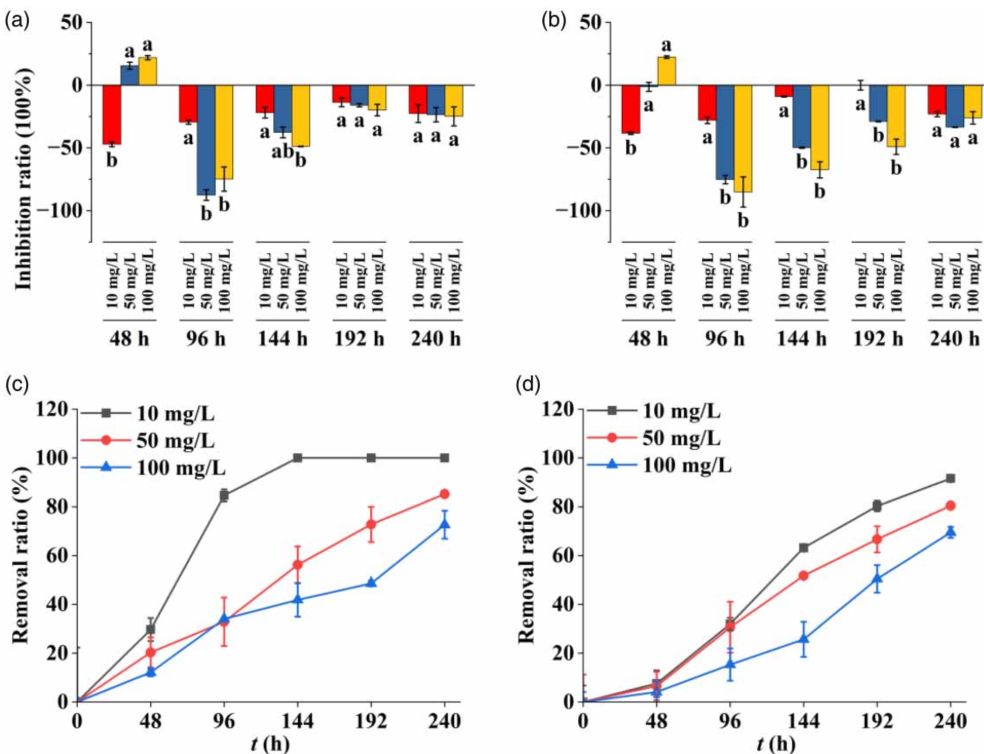

Figure 1 | The interactions of *Chlorella pyrenoidosa* with phenol and 4-fluorophenol: (a) the growth inhibition effect of phenol on *C. pyrenoidosa*; (b) the growth inhibition effect of 4-fluorophenol on *C. pyrenoidosa*; (c) the removal efficacy of *C. pyrenoidosa* on phenol; and (d) the removal efficacy of *C. pyrenoidosa* on 4-fluorophenol.

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3.2. The effect of phenol and 4-fluorophenol on *C. pyrenoidosa* ROS production and oxidative stress

The effects of phenol and 4-fluorophenol on the ROS and antioxidant enzymes of algal cells are shown in Figure 2. There was a slight variation in the ROS and SOD levels observed ($Value_{sample}/Value_{control}$ is close to 1) in response to phenol and 4-fluorophenol treatments. The soluble protein (SP) contents in the phenol and 4-fluorophenol treatment groups were significantly higher than those in the control group, indicating a vigorous growth of algal cells compared to the control group. However, the CAT and MDA contents in the phenol- and 4-fluorophenol-treated groups were lower than in the control group.

ROS, which includes hydroxyl radical (OH), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), etc., is a typical product of cellular metabolism. Excess ROS can lead to oxidative damage and disturb cell metabolism. Antioxidant defence systems maintained by SOD, CAT, etc., may prevent cellular damage caused by ROS and maintain physiological homeostasis. CAT is an inducible enzyme and ROS induces its gene promoter. However, the production of inducible CAT always lags behind ROS production. Therefore, the oxidation caused by ROS cannot be eliminated by CAT, leading to the production of MDA. The low levels of ROS, CAT, and MDA in the intracellular algal cells in the phenol and 4-fluorophenol treatment groups were stable and healthy compared with the control group. This is consistent with the results of the growth inhibition test, in which it was shown that the growth of algal cells after 96 h of treatment could be significantly promoted by phenol and 4-fluorophenol.

3.3. The effects of phenol and 4-fluorophenol on the metabolomics of *C. pyrenoidosa*

The metabolites of *C. pyrenoidosa* were investigated to further explore the effects of phenol and 4-fluorophenol on the growth of algal cells. Thirty-eight upregulated and 22 downregulated metabolites were screened in the phenol-treated group using *t*-test analysis. The conditions of a fold change of < -2 or > 2 and a p-value of < 0.05 were observed. At the same time, 34 upregulated metabolites and 12 downregulated metabolites in the 4-fluorophenol-treated group were screened. The clustering analysis of these metabolites is shown in Figure 3(a) and 3(b). It was found that there were 34 common differential metabolites between the phenol- and 4-fluorophenol-treated groups. More metabolites were upregulated than downregulated in phenol or 4-fluorophenol, indicating that most physiological activities were stimulated in *C. pyrenoidosa*. Differential metabolites of the phenol- and 4-fluorophenol-treated groups were used in metabolic pathway enrichment analysis and the results of this analysis are shown in Figure 3(c) and 3(d). Algae in the phenol- and 4-fluorophenol-treated groups were significantly affected regarding porphyrin and glycerophospholipid metabolisms.

Generally, the generation of photosynthetic pigments is closely related to microalgal growth (Liu *et al.* 2021). Photosynthetic pigments are the material basis for photosynthesis in plants and microalgae, and their content is considered a sensitive parameter under environmental stress conditions (Qian *et al.* 2018). As the most abundant universal chlorophyll pigment in plants, algae, and cyanobacteria, Chl-a is a valuable parameter that reflects the efficiency of light absorption by algal cells (Sutherland *et al.* 2015), and its biosynthesis is heterogeneous (Ioannides *et al.* 1994). The biosynthesis of chlorophyll in algal cells occurs via multiple and parallel biosynthetic routes and results in the formation and accumulation of various intermediate or transform products. Porphobilinogen is a pyrrole derivative essential for magnesium protoporphyrin synthesis. Chlorophyllide-a is the biosynthetic precursor of chlorophyll-a. 7-Hydroxychlorophyllide is an intermediate

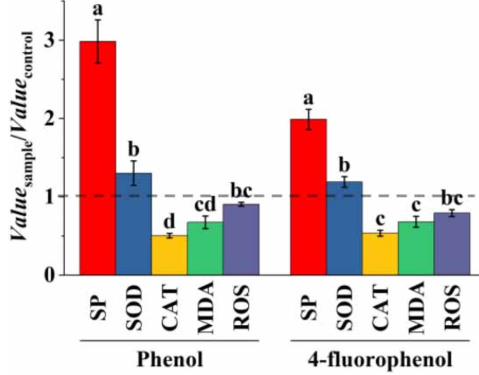

Figure 2 | Antioxidant enzyme activity and ROS levels of *Chlorella pyrenoidosa* treated for 96 h with 100 mg/L of phenol and 4-fluorophenol. SP, soluble protein.

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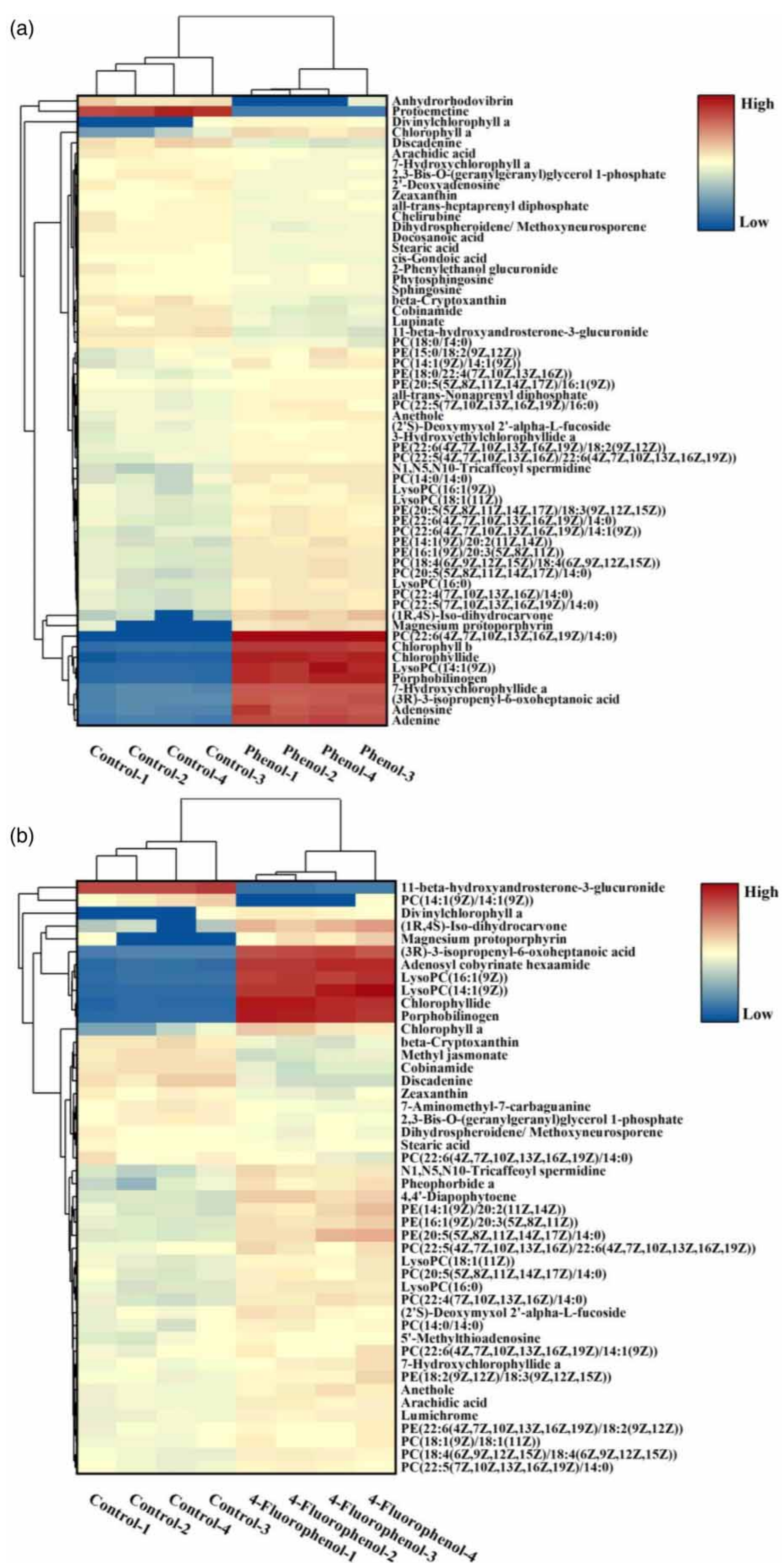

Figure 3 | Metabolomic analysis of *Chlorella pyrenoidosa* treated with each 100 mg/L of phenol and 4-fluorophenol for 96 h: (a) heat map of the differential metabolites of the phenol-treated group; (b) heat map of the differential metabolites of the 4-flouophenol-treated group; (c) bubble diagram of metabolic pathway analysis of the phenol-treated group; and (d) bubble diagram of metabolic pathway analysis of 4-fluorophenol-treated group. (*continued*.).

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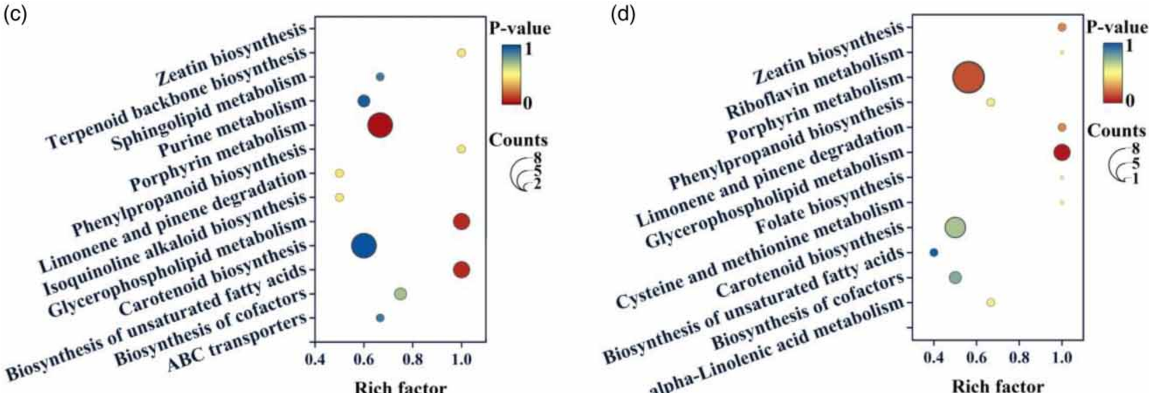

Figure 3 | Continued.

product of the transformation of chlorophylls a and b (Yang *et al.* 2016). Divinyl chlorophyll a and b are significant chlorophylls in the euphotic zone of tropical waters (Rebeiz *et al.* 1994).

Porphobilinogen, magnesium protoporphyrin, chlorophyllide, chlorophyll a, divinyl chlorophyll a, and 7-hydroxychlorophyllide a were upregulated in the phenol- and 4-fluororphenol-treated groups and chlorophyll b, 3-hydroxyethyl chlorophyllide a were upregulated in the phenol-treated group compared to the control group. In contrast, pheophorbide a was upregulated in the 4-fluorophenol-treated group. All these upregulated metabolites indicated that phenol and 4-flourophenol could significantly induce the biosynthesis of photosynthetic pigments, leading to the growth promotion of algal cells. It was reported that pharmaceuticals and personal care products (PPCPs) like ibuprofen, tylosin, erythromycin, and sulfamethoxazole could increase the content of chlorophyll a in algal cells, and the phenomenon was possibly attributed to compounds containing nitrogen atoms that can provide a nitrogen source or hormesis effect (Pinckney *et al.* 2013; Ding *et al.* 2017; Zhang *et al.* 2020). Considering that there is no nitrogen in phenol or 4-fluorophenol formulas, the hormesis effect may be the possible reason for the upregulation of chlorophyll a and its related compounds in the treated groups in this study.

Lipids are involved in regulating the response of organisms to external stress (Upchurch 2008; Chen *et al.* 2021c). For algal cells, membrane components (e.g., glycerophospholipids, betaine ether lipids, glycosyl glycerides, and phosphoglycerides) and storage lipids (mainly triacylglycerols) are the major lipid classes (Li-Beisson *et al.* 2019). There were 10 kinds of phosphatidylcholine (PC), eight kinds of phosphatidylethanolamine (PE), and four kinds of lysophosphatidylcholine (lysoPC) screened in this study as differential metabolites in the phenol-treated group, and all of these glycerophospholipids were upregulated. Similarly, there were 19 differential glycerophospholipid metabolites (10 kinds of PC, five kinds of PE, and four kinds of lysoPC), and 17 of the 19 compounds were upregulated in the 4-fluorophenol-treated group. In general, glycerophospholipids are metabolites of the average growth of algal cells. Changes in that composition can severely alter membrane fluidity, thickness, packing, and the dynamics and functions of membrane proteins (Hu *et al.* 2015; Huang *et al.* 2015). Unsaturated fatty acids are essential components of glycerophospholipids. Due to the antioxidant capacity attributed to their electron-donating ability (Huang & Wang 2004), the increase of unsaturated fatty acids benefits cell metabolism, which likely plays a role in stimulating cell growth.

Due to hydrogen and fluorine atoms being sterically quite similar, with their Vander walls radii being 1.2 and 1.35 Å, fluorine is one of the most classical bioisosteric replacements of hydrogen (Lima & Barreiro 2005). However, the electronegativity of fluorine is greater than that of hydrogen. Hydrogen replacement with fluorine could alter the physicochemical and biological activity of organic compounds. Each 100 mg/L of phenol and 4-fluorophenol can significantly stimulate the intracellular metabolism of *C. pyrenoidosa* in this study. The upregulation of photosynthetic pigments and glycerol phospholipids is the common essential mechanism of these two compounds to promote the growth of algal cells. ANOVA, combined PCA, and OPLS-DA analyses were conducted to explore the metabolite differences among all treated groups. The loading plots and cluster analysis are shown in Supplementary material, Figure S2(a)–S2(c). Most metabolites of the phenol and 4-fluorophenol-treated groups had the same change trend compared to the control group. Namely, after 100 mg/L of phenol or 4-fluorophenol treatment for 96 h, *C. pyrenoidosa* showed similar tolerance and metabolic response.

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4. CONCLUSION

The tolerance of *C. pyrenoidosa* to the substrates of phenol and 4-fluorophenol had a dose-dependent and time-dependent relationship, but the difference caused by the substrates was not apparent. In general, *C. pyrenoidosa* can grow well in $\leq$100 mg/L phenol or 4-fluorophenol exposure, while the removal efficiency of phenol or 4-fluorophenol by algal cells decreases with an increase in the exposure dose. The removal efficiency of 10 or 50 mg/L phenol or 4-fluorophenol by algal cells is higher than that of 100 mg/L. Anyhow, 100 mg/L phenol or 4-fluorophenol-treated by *C. pyrenoidosa* for 240 h had more than 70% removal efficiency. Interestingly, the growth of algal cells showed a growth trend of first inhibition and then promotion. After 96 h treatment, the SOD, CAT, MDA, and ROS levels of the phenol- and 4-fluorophenol-treated groups were similar or even lower than those of the control, proving that the growth of algal cells is stable after phenol or 4-fluorophenol exposure. Furthermore, it was revealed by a metabolomics study that significant upregulation of photosynthetic pigments and phospholipids could be caused by phenol and 4-fluorophenol treatment, benefitting the photosynthesis, antioxidation, and membrane stability of algal cells. Hence, it is indicated by these findings that *C. pyrenoidosa* is a promising biological material to remove such contaminants.

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AUTHORS CONTRIBUTION

M.L. conceptualized the study, prepared methodology, acquired funds, wrote the original draft. L.M. investigated the study, did formal analysis, wrote the original draft. Y.A. led the investigation, did data analysis. D.W. wrote the review and edited the file, acquired funds. H.M. conceptualized and supervised the study. J.Z. and C.W. prepared the methodology and supervised the study.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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