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Multistage defense response of microalgae exposed to pharmaceuticals in wastewater

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ABSTRACT

Recently, the use of microalgae for bioremediation of pharmaceuticals (PhAs) has attracted increasing interest. However, most studies focused more on microalgae removal performance, its defensive response to the PhAs during wastewater treatment remains unexplored. Herein, microalgal three defensive systems have been investigated in synthetic wastewater, with six PhAs as the typical drug. Results show that PhAs could bind to EPS, and this action in turn could help to alleviate the direct toxicity of PhAs to microalgae. Subsequently, the physiological analyses revealed the increase of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities, potentially reducing the oxidative stress induced by PhAs. Furthermore, the enzyme activities of cytochrome P450 (CYP450) and glutathione-S-transferase (GST) were significantly upregulated after exposure to SMX, CIP and BPA, followed by a significant decrease in biodegradation rates after the addition of CYP450 inhibitors, suggesting that the biotransformation and detoxification of PhAs occurred. Meanwhile, molecular docking further revealed that CYP450 could bind with PhAs *via* hydrogen bond and hydrophobic interaction, which proved their abilities to be metabolized and form transformation products in microalgae. These findings provide an advancing understanding of microalgae technologies to improve the treatment of wastewater contaminated with PhAs.

In recent years, pharmaceuticals (PhAs) are ubiquitously present in various wastewater, and the concentrations of PhAs have increased from the level of ng/L to mg/L, such as the tetracycline (0.69 mg/L) in livestock wastewater and ciprofloxacin (6.5 mg/L) in hospital wastewater [1–3]. Most conventional biological wastewater treatment techniques could only partially remove PhAs, an efficient and environmentally friendly method is thus required to better degrade these PhAs. Compared to mainstreamed biological technologies like activated sludge, microalgal wastewater treatment attracts increasing attention due to wide environmental adaptability and satisfactory removal efficiency [4–6]. Particularly, using microalgae to treat pharmaceuticals (PhAs) recently provides an opportunity to reduce the spread of super bacteria to meet the need of upgraded wastewater treatment [7,8]. However, the microalgal removal of PhAs from wastewater remains a challenge, because its bioremediation ability may be reduced even blocked with time due to the morphological and physiological changes caused by some PhAs [9]. Presently, only a few studies dedicated to identifying the potential impacts of PhAs on microalgal wastewater treatment, thereby an accurate reflection of microalgal treatment mechanisms is still limited [10]. However, understanding the interactions between microalgae and the PhAs in wastewater is critical to extend the biotechnological potential of microalgae for PhAs removal.

To date, the interactions between microalgae and the PhAs were intensively assessed with evaluating the toxicological effects in microalgae [11]. Generally, PhAs can inhibit chlorophyll production, membrane lipid peroxidation, and reactive oxygen species (ROS) formation, thereby exerting adverse effects on microalgal photosynthetic activity, growth, and cell viability [12,13]. However, many of these studies mainly investigated the cellular toxicity and physiological response under environment-irrelevant high concentration of PhAs, more less ignoring the contribution of microalgae towards PhAs bioremediation in the more actual situation [14]. Therefore, from the perspective of wastewater treatment (PhAs usually ranged from ng/L to few mg/L), whether and how low-level PhAs influence microalgae with its bioremediation roles are of grand necessity to explore. The low concentration of PhAs is generally considered with negligible impact on the growth of microalgae, but it can disturb the metabolic activity of microalgae [15]. A recent study has demonstrated that the quaternary ammonium compounds had negative impact on microalgal growth and could change microalgal bioactivity even at low micrograms per liter, consequently decreasing the removal efficiency of ammonia nitrogen and total phospho-

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rus [16]. Thus, an in-depth understanding of the microalgal adaptive response to PhAs stress is the key to further develop microalgal wastewater treatment system.

Microalgae can generally activate 'moderate' defense response to maintain their growth and keep it alive under unfavorable circumstance, and the microalgal resistance response to PhAs stress can be triggered either intracellularly or extracellularly [17,18]. In particular, extracellular polymeric substances (EPS) have a role in cellular protection *via* adsorption with pollutants [19]. Moreover, it is well-known that microalgal species can become more resistant to PhAs through antioxidant defense enzymes, which can present an important adaptive mechanism of microalgal cells to PhAs [20]. Furthermore, relevant literature suggested that various intracellular enzymes (CYP450s and GSTs) could act with the detoxification mechanism of organic compounds [21,22]. However, although some studies worked on microalgal defense response are informative on understanding the relationship between microalgal biological activity and PhAs, the underlying mechanism remains obscure. As far as we know, the detailed information about how microalgae defensively respond to the presence of PhAs is still lacking. Additionally, another key issue remained unexplained is whether the defense mechanism in microalgae will be different due to the structure and physicochemical properties of PhAs.

Thus, this work is to elaborate the mechanism about the multistage PhAs defense system in microalgae, and six typical and widely detected PhAs in wastewater were chosen. The specific objectives involve (1) evaluating the physiological bio-indicators (growth and photosynthesis) of microalgae to PhAs, (2) investigating the role of EPS as the first parclose defense for interacting with PhAs, (3) elucidating the impact of PhAs on the oxidative stress and antioxidant defense system in microalgae, and (4) revealing the mechanism for the PhAs-induced enzyme defense response and PhAs transformation through both experiments and molecular docking. Altogether, this work will shed light on tolerance and defense strategies of microalgae towards PhAs.

The experiments were conducted in the photobioreactor (PBR), which involved six PhAs (Table S1 in Supporting information lists their structural and physicochemical properties) treatment groups with 1 mg/L in sterilized artificial wastewater (wastewater composition is listed in Table S2 in Supporting information) for 10 days to assess microalgae physiological responses and PhAs removal. Without the PhAs spiked was set the control group. The cultivation was placed in PBR at 30 °C under 200 mmol m^{-2} s^{-1} illumination, and CO_2 levels maintained at 2.0% by continuous supply at a flow rate of 0.5 vvm. All the batch experiments were repeated in triplicate.

Additionally, a series of batch experiments were conducted to investigate the bioadsorption and biodegradation of PhAs in microalgae (Text S2). The biosorption of EPS in the removal process of PhAs was investigated by inactivated microalgae (with sodium azide-NaN_3). The cytochrome P450 inhibition (1-aminobenzotriazole, ABT) experiment was set to determine the role of CYP450 in the biotransformation of PhAs [23]. Similar cultivation conditions were consistent with the above. All the batch experiments were repeated in triplicate. During the experiments, the quantitative analysis for the six PhAs was measured using the Agilent 1260 high-performance liquid chromatography (HPLC, USA) (Detail see Text S3 in Supporting information).

The growth of *C. sorokiniana* was determined using a UV-vis spectrophotometer at the optical density of 680 nm (UV2600, Shimadzu, Japan). The biomass (g/L) was calculated following the curve between dry cell weight (DCW) and absorbance at OD680:DCW (g/L) = 0.125 × OD680 (R^2 = 0.992). Chlorophylls and carotenoids were determined with a UV-vis spectrophotometer at 440, 645, and 663 nm, respectively. And the chlorophyll fluorescence was measured using an AquaPen fluorimeter (PEA, Hanstech, U.K.) (Detail see Text S4 in Supporting information).

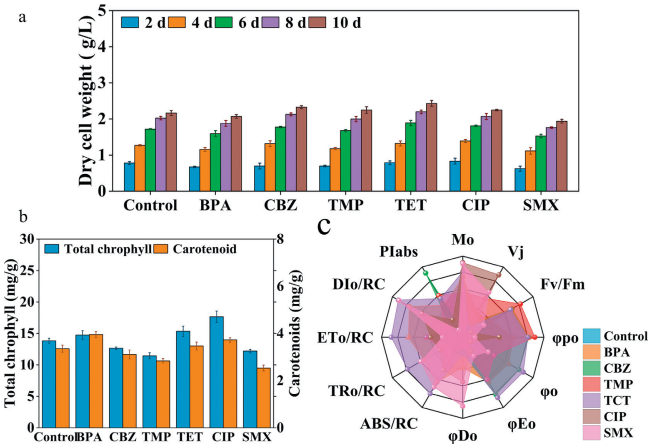

Fig. 1. Cell growth (a), total chlorophyll and carotenoid (Car) content (b), and PSII photochemical parameters of *C. sorokiniana* after exposure to PhAs (c).

The EPS of microalgae samples were extracted as per the modified heat extraction method. The soluble proteins and polysaccharides concentrations of EPS were assayed using the Bradford method and the anthrone-sulfuric acid method, respectively. The were measured using a fluorescence spectrometry (F-7000, Hitachi, Japan) (Text S5 in Supporting information). Scanning electron microscopy (SEM, TM3030Plus, Hitachi, Japan) was used to observe the cell morphology (Text S6 in Supporting information).

The distribution of ROS was stained by a fluorescence probe (2,7-dichlorofluorescin diacetate) and then observed was using a fluorescence microscope (ECLIPSE Niu, Nikon, Japan). Meanwhile, the fluorescence intensity was quantified by fluorescence spectrophotometry (F-7000, Hitachi, Japan). The antioxidant (SOD, CAT, and POD) and degrading (CYP450 and GST) enzymatic activities in cell were determined according to the corresponding kit according to the previous studies methods (Solarbio, Beijing, China) (Text S7 in Supporting information) [24].

The crystal structure of CYP450 enzyme in *C. sorokiniana* was prepared by SWISS-MODEL (http://swissmodel.expasy.org/), which the primary amino acid sequence was downloaded from NCBI (PRW58849.1). The 3D structures of ligands (six PhAs) were obtained from the Pub Chem database (https://pubchem.ncbi.nlm.nih.gov/), and GaussView 5.0.9 was used to optimize its structure. Then, the binding of PhAs to the CYP450 enzyme was investigated using Auto Dock 4.2, and each simulation process performed 200 runs [25]. The best conformation interactions were analyzed visually by the PyMOL program (Text S8 in Supporting information).

Fig. 1a shows the cell growth was slightly inhibited by PhAs, whereas the hormesis effect compared to control can be found in TET, showing that microalgae would have good resistance to these PhAs. This result was consistent with the previous studies showing that the growth of microalgae was inhibited at most PhAs levels [26]. Additionally, considering the photosynthetic physiological response shown in Fig. 1b, the total chlorophyll contents were significantly reduced with PhAs, indicating that PhAs could inhibit the biosynthetic pathway of chlorophylls in microalgae. This suggests that PhAs could further lead to chloroplast membrane peroxidation and induce the higher oxidative stress. Furthermore, the significantly increment of carotenoids (Car) in PhAs treatment was observed, suggesting Car would be a contributor against the PhAs stress. As known, Car are non-enzymatic antioxidants with strong antioxidant activity, which can eliminate the cellular ROS levels in microalgae [27]. Further, to specifically concern the PSII system and electron transport under PhAs exposure, a chlorophyll fluorescence was conducted in Fig. 1c [28]. The result shows that the inhibition degree of chlorophyll fluorescence yield parameters under

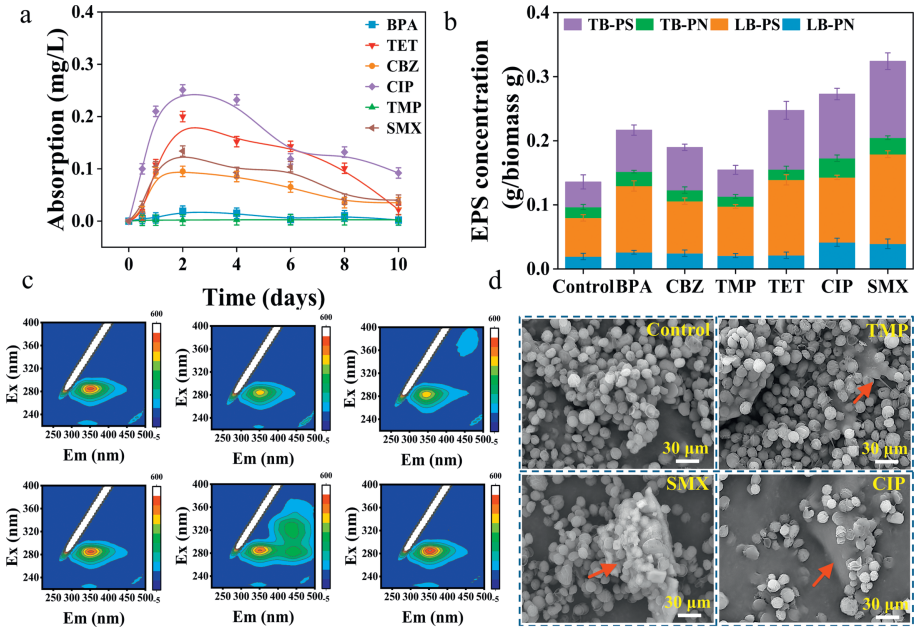

Fig. 2. Biosorption of PhAs by EPS (a), EPS compositions, including tightly bound protein/polysaccharide (TB-PN/PS) and loosely bound protein/polysaccharide (LB-PN/PS) (b), and 3D-EEM spectra of EPS after exposure to PhAs (c); cellular morphology of *C. sorokiniana* after PhAs exposure (d).

PhAs followed this trend CIP > SMX > CBZ > BPA > TMP > TET. Overall, these results indicate that low PhAs concentration could partially disturbed microalgal physiological status, but the inhibition level varied with different PhAs.

EPS secreted *via* microorganism is considered as a vital role-player for cell protection [29,30]. To investigate the role of EPS, a PhAs adsorption test was conducted to further scrutinize the degree of adsorption by inactivated microalgae (with NaN_3). Shown in Fig. 2a, the concentrations of PhAs in EPS increased faster on the first day and then gradually reduced, which may suggest the contaminants were bio-uptaken by microalgal cells through EPS [31]. Furthermore, EPS had nearly no adsorption for TMP and much lower adsorption efficiency for BPA and CBZ, which is similar to the literature reported in activated sludge system [32]. The precise reason for this discrepancy was still unclear but would be related to the different physicochemical characteristics of PhAs. For instance, CIP and TET would exist as the anionic or zwitterionic form in wastewater (pH of 6.5-7.5), which is superior for forming an electrostatic interaction with the negatively charged surface of microalgae [33,34]. Additionally, the different concentration and characteristics of EPS were observed in microalgae, which would be another factor to influence PhAs adsorption (Fig. 2b). Specifically, microalgae induced by CIP and SMX produced more EPS, which was 2-fold higher than TMP. Polysaccharides in EPS exhibited obviously larger quantity than proteins, indicating that polysaccharides had a greater binding affinity with PhAs. This is because the multiple functional groups such as –CH, –OH, and hydrophobic moieties contained in polysaccharides contain are conducive to the formation of CH-π bond and hydrogen bond with PhAs [35,36]. It is well known that the proteins in EPS are a mixed substance, while 3D-EEM can conveniently depict the main fluorescent substance. As shown in Fig. 2c, two protein peaks of EPS extracts (Ex/Em = 275-310 and 230-326) detected by 3D-EEM displayed a fluorescence intensity order of CIP > SMX > TET > CBZ > BPA > TMP, which is consistent with Fig. 2a. These results proved that the tyrosine/tryptophan and aromatic proteins would also play important roles in the adsorption of PhAs. Meanwhile, the EPS could usually promote microbial aggregation. For further characterization, the cellular morphology during the PhAs exposure was analyzed by SEM (Fig. 2d). Compared with control, some microalgae cells showed a slightly compact configuration after TMP exposure, while the agglomeration was noticeably appeared after CIP and SMX stimulation. Compared with control, some microalgae cells showed a slightly compact configuration after TMP exposure, while the agglomeration was noticeably appeared after CIP and SMX stimulation. This demonstrates that EPS would contribute to the intermolecular association and microalgae aggregation, which can also preferentially benefit PhAs biosorption. Overall, the increased EPS content would be capable of alleviating the direct toxicity of PhAs to microalgae.

Most PhAs are rapidly bioadsorbed through EPS and intracellularly transformed. The bioaccumulation of PhAs in microalgae can induce the generation of ROS, and the excessive ROS would lead to severe cell damage and subsequently suppress cell growth [37]. In Fig. 3a, the content of ROS was slightly increased compared with control, wherein similar ROS contents were observed with the different PhAs treatments. Fig. 3b further indicated that the cell emitted yellow fluorescence under 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), thus proving the increased generation of ROS by PhAs. Moreover, microalgal cells with fluorescence were agglomerated after CIP and SMX exposure (Fig. 2d). As known, the ROS induced oxidative damage can be mitigated through the antioxidant defense enzymes of microalgae, in which SOD, POD, and CAT are believed as the most powerful antioxidative enzymes used to protect cells throughout a series of chain reactions to eliminate ROS [38]. Here, we identified the expression of these enzymes was significantly altered in PhAs relative to control (Fig. 3c). To be specific, SMX remarkably improved the activity of SOD, POD, and CAT, with the effect being more significant for POD. In contrast, the CAT activity was mainly disturbed as a result of CBZ and CIP exposure, which indicates that CAT would be the pivotal antioxidant enzyme to remove ROS. Both the TET and BPA significantly increased the activity of POD and CAT, clearly denoting as the tandem occurrence for ROS scavenging. In contrast, fewer antioxidant enzymes were induced by TMP stimulation, which is consistent with the photosynthetic performance shown in Fig. 1c. These foregoing results suggested that the antioxidant defense system was activated by PhAs *via* regulating the corresponding enzyme activity. Inter-

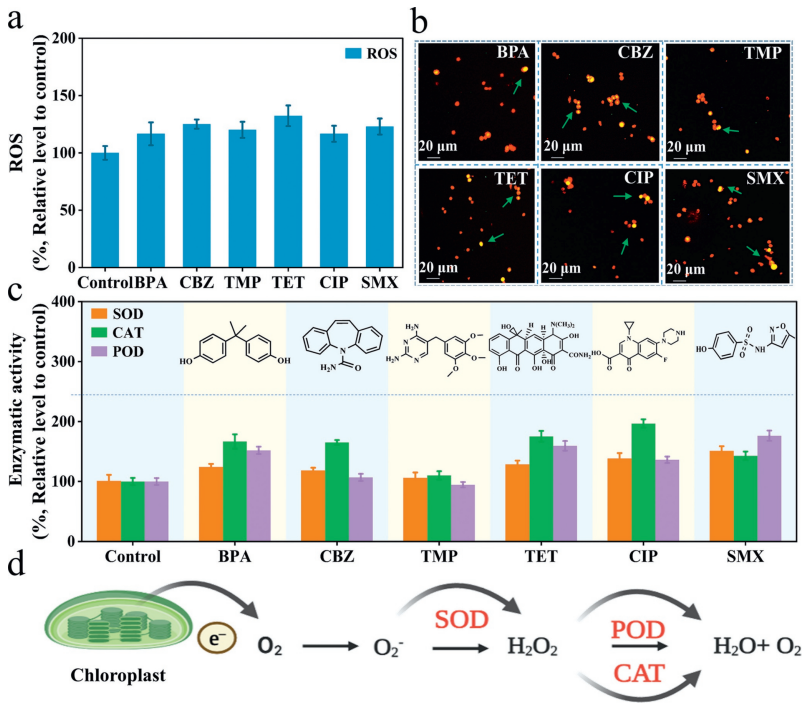

Fig. 3. ROS content in *C. sorokiniana* after PhAs exposure (a), and ROS expression *via* fluorescence microscopy (b); activity of antioxidant enzymes in *C. sorokiniana* after PhAs exposure (c); schematic diagram of the mechanisms on the antioxidant ROS removal system (d).

estingly, these antioxidant enzymes did not show a homogeneous impact of the PhAs in their antioxidant performance, implying that the catalytic action of antioxidant enzymes was unique. Generally, the intracellular ROS ($^{\bullet}O_2^-$, OH^-, and H_2O_2) can be transformed into hydrogen peroxide (H_2O_2) by SOD in cytosol, and further be converted as water and oxygen by POD and CAT located in peroxisomes (Fig. 3d) [39,40]. Microalgae showed diverse antioxidant patterns in the antioxidant defense system, which may also be highly dependent on PhAs structure. It was reported that the presence of methyl and carboxyl groups in organic compounds could reduce the generation of oxidative stress [41]. Taken together, the increased activities of the antioxidant enzymes can defensively protect the microalgae from ROS damage [42]. Furthermore, POD may play a crucial role in protecting microalgae to defend microalgae from oxidative damage, it has rarely been previously appreciated in microalgae.

Fig. 4a provides an overview of the activity of detoxifying enzymes (phase I and II) in microalgae exposed to various PhAs. As compared to control, the CYP450 activity was significantly upregulated, with the corresponding order of CIP > SMX > BPA > CBZ > TMP. However, TET did not influence CYP450 activity, as it can be mainly removed by photodegradation [43]. This indicates that the detoxification of CYP450 was activated to metabolize PhAs in microalgae. In addition, the activity of GST (a phase II detoxification enzyme) was also determined here, discovering that BPA and SMX induced the intensive increment in GST activity. Several studies reported that GST enzymes catalyzed PhAs' further biotransformation *via* conjugation with large and polar compounds (*e.g.*, sugars and amino acids). BPA glycosides and pterin-SMX formed from glycosylate conjugation have been commonly observed in microalgae as a detoxifying mechanism during PhAs exposure [44,45].

To investigate the contribution of CYP450 in PhAs biodegradation, an enzyme inhibitor (ABT) assay was employed. Gratifyingly, shown in Fig. 4b, the biodegradation rate of BPA, CIP, and SMX was significantly inhibited when CYP450 was inactivated. The observed result is consistent with the literature that SMX and CIP were catalyzed by CYP 450 in sulfate-reducing bacteria sludge [46]. In addition, less than 10% of the inhibition rate was observed in CBZ and TMP, showing that the low concentration did not strongly trigger the CYP450 response. Contrary to other PhAs, CYP450 nearly did not involve in the biodegradation of TET, which is consistent with the results in Fig. 4a. Together, the results indicated that for most of the biodegradable PhAs, enhancing the CYP450 metabolic activation may be the key factor in the microalgae-mediated degradation of PhAs.

To further understand the detoxification mechanism, herein, the catalytic process of CYP450 and PhAs was analyzed through the molecular docking simulation. As shown in Table S3 (Supporting information), the interaction energies of BPA, CBZ, TMP, CIP, and SMX ranged from −5.38 kcal/mol to −7.26 kcal/mol for CYP450, suggesting that the binding affinities varied markedly among different PhAs. Importantly, compared with the biodegradation assay, it seems to exist a good correlation between PhAs binding affinity and PhAs biodegradation rate (Fig. 4c). That is to say, the higher binding energy between CYP450 and PhAs may contribute to the occurrence of metabolic reaction, supporting the experimental observation that PhAs were more liable to be degraded (Fig. 4d). Moreover, we speculated that the molecular docking can provide a mechanistic understanding of how the molecular structure of PhAs influences their CYP450's catalytic behavior. Judging from the docking conformation, different binding orientations and action sites of PhAs with CYP450 may be the reason for the varied biodegradable rates. For BPA and CBZ that have similar structure and physicochemical properties, the interactions with CYP450 were substantially different (Figs. 4e and f). BPA was oriented toward the favorable orientation to the active center undergoing methyl hydroxylation [40], whereas the carboxamide group of CBZ was far from the active center that exhibited a catalytically unfavorable conformation. Concerning hydrogen bonding interaction, BPA formed more hydrogen bonds with the residues LYS71, ALA297, and THR250, only one instance of H bonding occurred between CBZ and CYP450, and the major residue participating was THR250. This

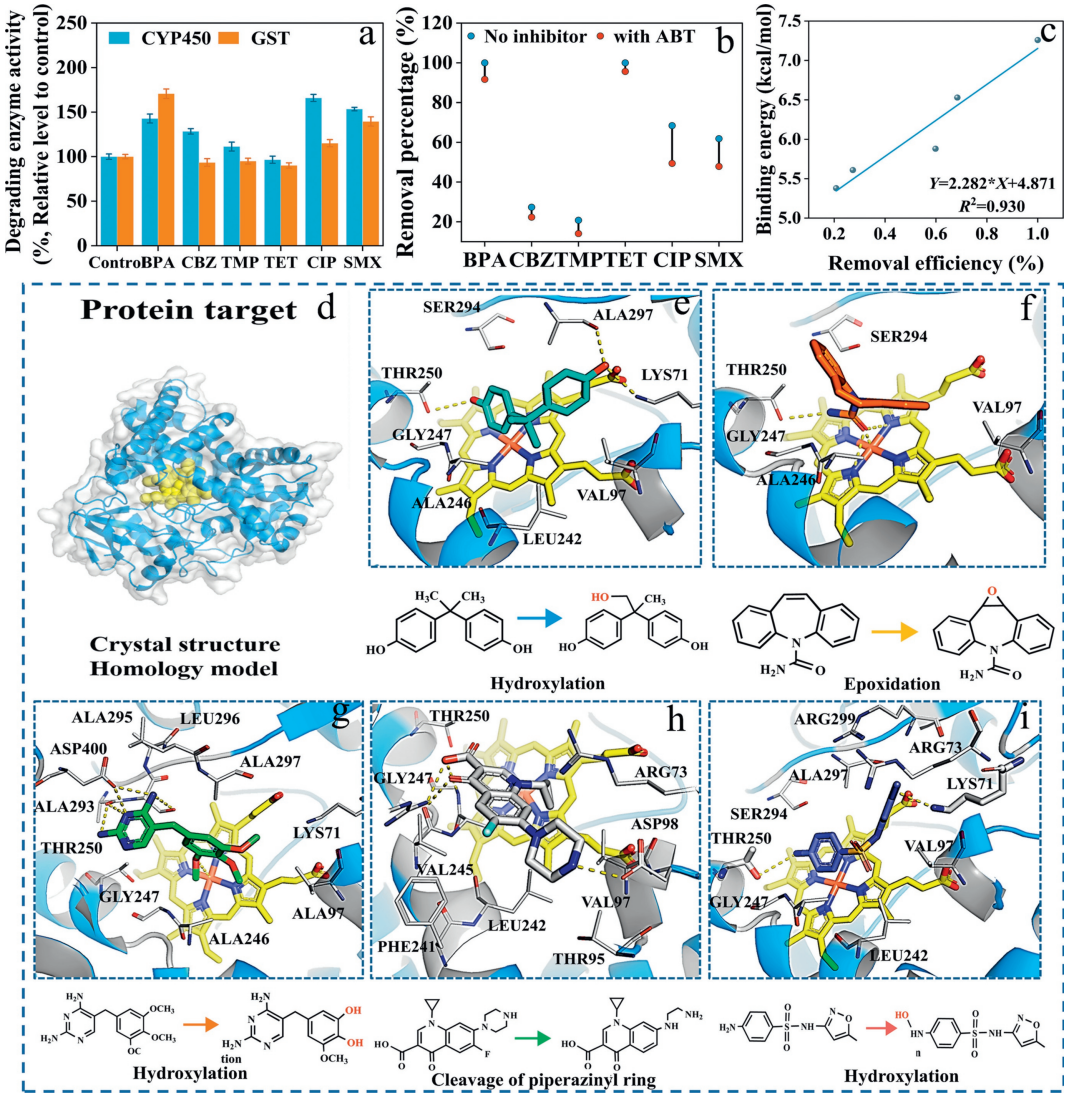

Fig. 4. CYP450 and GST enzyme activities in *C. sorokiniana* after PhAs exposure (a); comparison of removal rate between PhAs and PhAs+ABT (b); relationship between binding energy and removal efficiency of PhAs (c); 3D structure of CYP450 enzyme by homology modeling (d); key sites of PhAs binding to CYP450, and the corresponding transformation production of PhAs (e–i).

result confirms that BPA had a higher biotransformation rate than CBZ. For TMP, the methoxy groups were oriented toward the inner part of CYP450 and thus formed hydrogen bonds with the residues ALA293, SER294, and ASP400, which can produce corresponding di-hydroxylated products (Fig. 4g). However, the biodegradation rate of TMP was slow, which the large molecular volume may hinder its access to the active center. For CIP, it was docked into the active center toward the inner part of a binding pocket in CYP450, forming hydrogen bonds with the residues VAL245 and GLY247 and allowing CIP to fit into the binding site properly. Meanwhile, the piperazine ring of CIP was more favorably oriented toward the residue ASP58 to establish another hydrogen bond interaction (Fig. 4h), supporting that CIP may undergo a cleavage at the piperazine ring with the hydroxylation at the methyl pyrrolidine ring [47]. In addition, the high biodegradation rate of CIP may be due to the multiple reactive sites that can be catalyzed by CYP450 [48]. For SMX, the aniline ring was oriented toward the reactive center, which resulted in a sufficiently close distance for the hydroxylation at the metabolic site (Fig. 4i) [49]. Furthermore, the number of amino acid residues that hydrophobic interact with SMX was significantly increased, which might explain the distinct binding affinities compared with other PhAs (Table S3). These results demonstrated that the orientation and position of PhAs toward the active site are the most important factors for determining the catalytic metabolism of PhAs by CYP450. The interaction structures for the binding geometries are likely to be the main element explaining the different preferences of CYP450 enzymes to the PhAs.

This study comprehensively unravels the defense mechanisms of microalgae against PhAs in wastewater. The results indicate that the defense of microalgae under PhAs is a multistage continuous process involving bioadsorption (EPS), bioaccumulation (intracellular antioxidant system), and biodegradation (detoxifying enzymes) (Fig. 5). In addition, microalgae represent different biological effects under different PhAs exposures, which could be related to the physical and chemical properties and chemical structures. Mechanism studies reveal that the secretion of EPS by microalgae was the first line of defense to resist PhAs, the main components in EPS (proteins and polysaccharides) play an important role in binding to TET and CIP. Further analysis identified the activated antioxidant defense system SOD, CAT, and POD, which can relieve the ROS produced by SMX and BPA bioaccumulation. Finally, CYP450 and GST play a detoxification role by catalyzing intracellular PhAs

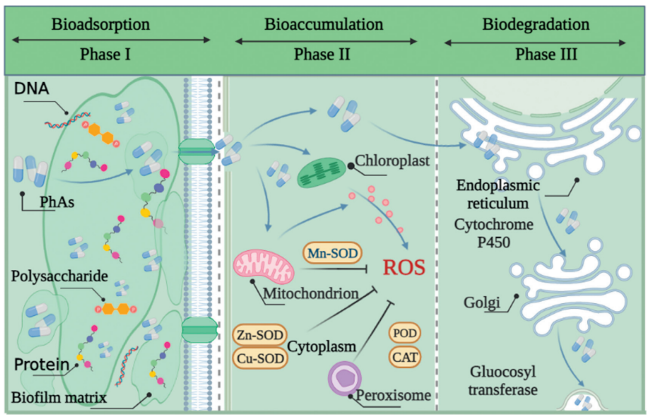

Fig. 5. Proposed mechanisms of removal of PhAs by *C. sorokiniana*, include biosorption, bioaccumulation, and biodegradation.

biotransformation (except for TET). Molecular docking further revealed that CYP450 enzyme and PhAs were closely bound, and the hydrogen bonds and hydrophobic interactions would highly contribute to the biodegradation of PhAs. These findings systematically provided insight on understanding the defense mechanisms that can provide useful information for targeted microalgae-based PhAs removal performances improvement.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.08.007.

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