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RESEARCH ARTICLE

Enhancement of the immobilization on microalgae protective effects and carbamazepine removal by *Chlorella vulgaris*

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Abstract

Carbamazepine (CBZ) has drawn extensive attention due to their environmental threats. In this study, polyvinyl alcohol-sodium alginate polymers to immobilize *Chlorella vulgaris* (FACHB-8) were used to investigate whether immobilization can facilitate microalgae to alleviate the CBZ stress and enhance CBZ removal. The results showed that after immobilized treatment, the biomass of microalgae increased by approximately 20%, the maximum level of malondialdehyde content decreased from 28 to 13 μmol/g, and the photosynthetic capacity of F_V/F_M recovered to 90% of the control group. The CBZ removal rate increased from 67 to 84% by immobilization at a CBZ concentration of 80 mg·L^{-1}. The results indicated that immobilization technology can effectively protect microalgae from CBZ toxicity and improve the removal of CBZ, especially at high concentrations (> 50 mg/L). Biodegradation was the dominant pathway for microalgae to remove carbamazepine. This study added the understanding of the microalgae responses under immobilization and the interactions between immobilized microalgae and CBZ removal, thereby providing a novel insight into microalgae technology in high concentration wastewater treatments.

Keywords Immobilized *Chlorella vulgaris* · Carbamazepine · Antioxidant response · Photosynthetic activity · Biological removal

Introduction

Pharmaceutical contaminants (PhCs) have become environmental pollutants of increasing concern due to their stability in water and potential toxicity to nontarget organisms (Meng et al. 2021). In a variety of pharmaceutical components, the most widespread psychotropic drug is carbamazepine (CBZ) (Archer et al. 2017). Carbamazepine is a first-generation anticonvulsant drug that has been used to treat seizures and other conditions related to the central nervous system and explosive usage for nearly 40 years (Garcia-Espinoza et al. 2018). Some studies have shown that CBZ is frequently excreted into aquatic environments an unmetabolized form by humans and animals following medical applications. Moreover, persistent improper utilization and incomplete treatment will exacerbate carbamazepine accumulation in the environment (Jarvis et al. 2014). The detection frequency of carbamazepine is more than 85% in aquatic environment worldwide, especially in North America, Europe, and East Asia (Villota et al. 2021). Due to its stable chemical properties and biological and photodegradation resistance, it has good bioaccumulation and long-distance migration potential (Song et al. 2019). CBZ had toxicological effects not only on primary organisms such as fungi, microalgae, and invertebrates but also on predators such as fish and humans that intake CBZ through the food chain. Moreover, CBZ with

Highlights
- Immobilization improved 6 to 17% of carbamazepine removal by *C. vulgaris*.
- ·Immobilization reduced oxidative stress and repaired photosynthesis in *C. vulgaris*.
- ·The protective effect of immobilization was more apparent at high CBZ stress.
- ·Biodegradation accounted for more than 80% of the total removal rate of carbamazepine.
- ·Immobilization improves the proportion of biodegradation in the CBZ removal pathway.

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low concentrations can inhibit the growth of human embryonic cells (Mojiri et al. 2020; Qiao et al. 2014).

To address the growing environmental threat posed by carbamazepine, a large amount of wastewater treatment technology has been studied (Meng et al. 2021). Among them, physical adsorption methods, advanced oxidation processes, and biological treatment technology are representative methods (Mojiri et al. 2020; Qin et al. 2016). Physical adsorption method commonly used the porous materials such as activated carbon and its modification as adsorbent; this method can improve the removal rate of CBZ. However, physical adsorption method had the disadvantage of reduced treatment effect after adsorption saturation in the treatment of high concentration pollutants and the treatment of saturated adsorbents also caused the environmental problems. Although AOP has better removal efficiency, it consumes a large amount of energy and operational cost (Kusmayadi et al. 2021); meanwhile, the byproducts produced in the processing will be more toxic than parent compound and pose a secondary environment threat (Zaied et al. 2020). As a result, these two technologies are limited by environmental impact and economic value and have not been widely promoted (Tang et al. 2020). Biological treatment technologies have different technical characteristics based on the different target organisms, among which the classic treatments are constructed wetland technology using macrophytes (Sharif et al. 2014) and membrane reactor technology domesticating microbial colonies (Yao et al. 2020). However, the long operation period and weak ability to resist impact loads are shortcomings that hinder the wide application of such technologies (Ferrer-Polonio et al. 2020). As an emerging biological wastewater treatment technology, microalgae biotechnology has become a potential efficient approach for the treatment of CBZ due to the slight environmental threat of metabolites, low operating costs, and the economic effect of recycling microalgae (Peter et al. 2021).

Microalgae, as versatile microorganisms ubiquitous in the environment and serve as primary producers in aquatic ecosystems, are often used as key indicators to evaluate water quality and pollutant toxicity (Sun et al. 2020). Microalgae possess the ability to remove environmental contaminants from aquatic environments, such as antibiotics, heavy metals, and flame retardants (Pan et al. 2021), and it can assimilate nitrogen, phosphorus, and inorganic compounds from wastewater and take advantage of optical energy to grow and metabolize (Chai et al. 2021). The process of pollutant removal by microalgae is divided into two parts, a portion of the pollutants are removed by biosorption, and the other part will be decomposed into micromolecule with less toxicity through enzyme metabolism after entering microalgae cells (Cheirsilp and Torpee 2012). In addition to removing target contaminants, microalgae biological treatment has two significant advantages over other technologies: (i) Microalgae can absorb carbon dioxide from the environment in the process of treatment, reducing the growing greenhouse effect (Almomani 2020b; Zhang et al. 2020). (ii) Microalgae, as green and sustainable biofuels, can effectively alleviate the pressure of energy shortages (Nagappan et al. 2020; Park et al. 2016). However, there is still some potential for improvement in microalgae wastewater treatment technology. First, exposure of microalgae to pharmaceutical contaminants inhibits growth rates and damages antioxidant systems in microalgae cells (Hena et al. 2021). Microalgae cell damage may occur, and biochemical functions may be disrupted. The mass transport capacity and the photosynthetic conversion efficiency were weakened, which further affected the biodegradation capacity of microalgae. Second, due to the small size and massive dispersion in the aquatic environment, the separation and harvesting of microalgae may account for 20–30% of the total production cost, and efficiently and economically extracting microalgae remains a challenge (Marangon et al. 2021). It was recently reported that the immobilization, referring to the entrapment or cross-bonding of microorganisms inside a specific substrate to make it more stable and reach some specific function, can be a helpful supplemental technology in microalgae wastewater treatment (Hena et al. 2021; Wu et al. 2021). The combination of immobilization technology with enzymes and bacteria has shown that it can effectively prolong the retention time of microorganisms in the environment and improve the ability to the resist environmental load through the interaction between substrate and extracellular polymeric substance (EPS) (Ashkan et al. 2021). The immobilized matrix can effectively solve the problems of microalgae separation and recovery (Zhuang et al. 2020). However, information about whether immobilization could help microalgae avoid oxidative damage and remove CBZ is still fragmental.

In this study, we used polyvinyl alcohol-sodium alginate (PVA-SA) polymers to immobilize microalgae to study the toxicity of microalgae and the removal of carbamazepine. *Chlorella vulgaris*, a type of mixotrophic microalgae, has the characteristics of simple culture conditions, high growth rate, large specific surface area, and strong adaptability, so it is widely used in microalgae research as a model organism (Jaiswal et al. 2021; Karim et al. 2021; Silambarasan et al. 2021). And it was suggested as a potential species to remove PhCs (Song et al. 2019). Therefore, *Chlorella vulgaris* (FACHB-8) was selected as the target microalgal species in this study. The main objectives of the present study were to explore (1) whether the immobilization can alleviate the toxicological effects of CBZ on *Chlorella vulgaris* based on their growth, photosynthetic capacity, antioxidant system response, and cellular damage (MDA content), and then (2) whether immobilization can enhance the removal of CBZ by

Chlorella vulgaris. The biological removal mechanisms of free microalgae and immobilized microalgae were compared and discussed. This study will provide an understanding on the CBZ removal by microalgae wastewater treatment combined with immobilization technology.

Materials and methods

Microalgal strain and cultivation procedure

Chlorella vulgaris FACHB-8 was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and cultured in BG-11 medium in a 250-ml flask. The culture was placed in a light incubator (DGX-350B, Safu Experimental Technology, NingBo) at a light intensity of 5000 lux with a light/dark ratio of 12:12 h at a temperature of 25 °C. The CO_2 concentration in the reactor can be maintained at 1–1.5%, which can meet the basic requirements of stable growth of *Chlorella vulgaris*. Cultured algae were treated with CBZ during the exponential growth phase.

Microalgal immobilization

Chlorella vulgaris cells were entrapped in PVA-SA beads as follows: First, microalgae were harvested from the culture medium (BG-11) via centrifugation at 3000 rpm for 10 min; after discarding the supernatant, the pellet was washed three times using 0.85% NaCl sterile solution (w/v) and resuspended in the same NaCl solution. Twenty milliliters of 2% sodium alginate and 2% polyvinyl alcohol solution was prepared and used after heating at 98 °C and cooling to room temperature. Then, 5 mL of the *C. vulgaris* suspension was mixed with 20 mL of 2% sodium alginate and 2% polyvinyl alcohol solution, and the mixture was gently stirred for 15 min. The resulting solution was dropped in 2% $CaCl_2$ and 3% H_3BO_3 solution (w/v) using a sterile syringe for the formation of uniform algal beads. The formed beads (the average diameter is 4mm) were left for 12 h at 4 °C for curing and then washed twice in a sterile saline solution (0.85% NaCl). The cell density of PVA-SA beads was approximately 2×10^7 cells mL^{-1} for every PVA-SA beads.

Experimental setup

The batch experiments were conducted in 250-mL flasks containing 200 mL of BG-11 medium in a light incubator. All incubations were cultivated under the conditions described in the "Microalgal strain and cultivation procedure" section. The experiment contained two experimental conditions: immobilized *C. vulgaris* by PVA-SA beads and free *C. vulgaris*. The initial concentration of *Chlorella vulgaris* was approximately 2×10^7 cells mL^{-1} (OD680= 0.21). Each treatment was divided into five groups based on different CBZ concentrations: the initial concentrations were set at 10, 35, 65, and 80 mg/L, and the no-CBZ groups were used as a control check (CK). The CBZ concentrations were chosen on the basis of the previous report. Several previous studies have reported the 50% microalgal growth inhibition concentration (EC50) was during the concentrations of 0 to 100mg/L (Chong et al. 2021; Xiao et al. 2021). Therefore, the concentration gradient is selected for analysis and research. The microalgae biomass in each treatment of three replicates was collected at 0, 2, 4, 6, 8, 10, and 12 days of cultivation to analyze the growth trend, antioxidation enzymes, cell damage, and photosynthesis characteristics. The concentration of residual CBZ in water was assessed daily using water samples (1 mL) collected from the needle sampler.

Analysis

The biomass of *Chlorella vulgaris* was measured according to the method used by Almomani (Almomani 2020a). The specific growth rate (μ) was measured by fitting the dry cell weight (DCW, $\mu g \cdot L^{-1}$) to an exponential function, which was established using Eq. (1) as proposed by Kabra (Kabra et al. 2014):

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad (1)$$

where N_0 and N_1 represent the DCW at the start of the exposure (T_0) and the assessed exposure time (T_1), respectively.

A linear relationship was observed between the DCW of *Chlorella vulgaris* and the OD_{680}, as shown in Eq. (2):

$$\text{DCW of FACHB } 8 = 205.83 \bullet OD_{680} - 15.61, \quad R^2 = 0.99 \quad (2)$$

The measurement of total chlorophyll content was performed according to the method used by Kurade (Kurade et al. 2016), while malondialdehyde (MDA) and superoxide dismutase antioxidant responses were measured by assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) on the 12th day of the experiment. Measurement of photosynthetic activity was conducted using a portable fluorometer (FMS-2, Hansha Scientific Instruments, UK), and FluorPen v.1.0 software was used for data analysis.

CBZ concentrations were analyzed using an Agilent 1260 HPLC system (Agilent Technologies, US). The column temperature was maintained at 25 °C during sample analysis. The isocratic mobile phase consisted of acetonitrile and 0.5% acetic acid solution (45:55 v:v) at a flow rate of 0.8 mL/min. The detection wavelength was 284 nm, and the injection volume was 20

μL. The retention time for CBZ in the HPLC system was 4.8 min, and the detection limit was 0.02 mg/L.

The removal of CBZ was divided into four categories according to the method reported by Song (Song et al. 2019): biodegradation (B_d), bioaccumulation (B_a), biosorption (B_s), and abiotic removal (ΔR). The process of B_d, B_a and B_s determination is as follows: 1 mL of microalgae solution was centrifuged at 5000 rpm for 10 min. Then, the layer of algae solution was shaken for 5 min by adding 1 mL of methanol, and centrifuged at 5000 rpm for 10 min again, and the gathered supernatant was used for the determination of biosorption (B_s). In carbamazepine content, the microalgae solution was sonicated for 1 h, placed in a refrigerator at −20 °C for one night, then added 1 mL of dichloromethane and methanol mixture (v:v = 1:2), centrifuged at 5000 rpm for 10 min. The obtained supernatant was used for the determination of intracellular bioaccumulation content (B_a) (Song et al. 2020). The abiotic part (ΔR) in the immobilization was mainly by the adsorption of PVA-SA beads. The beads were removed from the solution, placed in a 10-ml methanol solution, and then centrifuged at 5000 rpm for 10 min. The gathered supernatant was used for the determination of the abiotic part (ΔR). The extraction process of microalgae in the beads was as follows: PVA-SA beads were first dissolved in 4% $NaHCO_3$ solution (w/v), and after the complete disintegration of beads, microalgae were obtained (Mujtaba and Lee 2017). Therefore, the total process was defined according to Eq. (3):

$$R = B_d + B_a + B_s + \Delta R \tag{3}$$

A pseudo-first-order kinetic model was used to describe the carbamazepine removal by the microalgae (Almomani and Bhosale 2021). Equation (4) is as follows:

$$\ln\left(q_e - q_t\right) = \ln q_e - k_1 t \tag{4}$$

where q_t refers to the carbamazepine removal concentration by microalgae at time t (mg/g); k_1 refers to the pseudo-first-order rate constant (day^{-1}); q_e refers to the removal equilibrium capacity (mg/g); t refers to the exposure time (day).

The significant differences in the growth rate, photosynthetic activity, and antioxidation enzymes of *C. vulgaris* under free and immobilized conditions were analyzed using one-way analysis of variance (ANOVA). The p value less than 0.05 was considered reliable. These calculations were conducted in Origin 9.0 software.

Results and discussion

Microalgal biomass growth

The biomass growth of free and immobilized *C. vulgaris* under different CBZ concentrations is illustrated in Fig. 1 a and b. The biomass of *C. vulgaris* followed the trend of gradual increase with time, in both the immobilized and free states. On the 12th day of the experiment, the OD_{680} value of the low concentration group (<50 mg/L) increased from 0.21 to 0.68, while the OD_{680} increment of the high concentration group (>50 mg/L) decreased by approximately 24% compared to the low concentration groups. The significant difference (p<0.05) was observed in free *C. vulgaris* biomass growth when exposed to low and high CBZ concentrations, indicating that CBZ significantly inhibited the growth of free *C. vulgaris* at high concentrations. However, after immobilization treatment, no significant inhibition of OD_{680} was observed in comparison to the treatments without CBZ. The biomass increments of *C. vulgaris* under high concentration decreased only approximately 8% lower than the low concentration group. These results indicated that the immobilization technique significantly alleviated the growth inhibition state of microalgae, especially at high concentrations. The reason for this phenomenon is that the substrate used for immobilization is wrapped outside the microalgae cells, and the migration of pollutants takes time, such that the concentration difference is formed and the microalgae are protected from direct exposure to the pollutant in the environment. From day 2 to day 8 of the exposure period, a portion of *C. vulgaris* growth rates were higher in the CBZ exposure groups than in the control group. The phenomenon of low CBZ concentrations stimulating growth might be explained by the hormesis effect, which has been widely reported in previous studies (Vo et al. 2019a). This effect is not apparent at high concentrations because microalgal cells may be damaged, leading to growth inhibition.

As shown in Fig. 1 c and d, the special growth rate decreased gradually with increasing CBZ concentration in both free and immobilization states, suggesting CBZ had inhibition effects on *C. vulgaris*. Compared to the continuous inhibition effect on free *C. vulgaris* throughout the culture period, it was found that the inhibition effect of CBZ on the immobilized *C. vulgaris* was weakened with increasing culture time. Under high CBZ concentration exposure, the special growth rate (μ) of immobilized *C. vulgaris* gradually increased from 50 to 85% of the control group. On the 12th day, the *C. vulgaris* growth rate was essentially equal to the control group. This phenomenon is caused by the mechanisms that microalgae use nutrients to produce proteins, lipids, polysaccharides, and nucleic acids to grow biomass (Almomani 2020a, 2020b). They need to convert light energy into biomass through photosynthesis, respiration, and the Calvin cycle and store it in cells for growth (Noguchi et al. 2021). However, under the environmental threat of PhC pollutants, the growth rate of microalgae decreased with decreasing energy conversion capacity (Quinn and Davis 2015; Zhang et al. 2019). Hence, the decrease in the inhibition effect of the growth rate indicated that immobilization

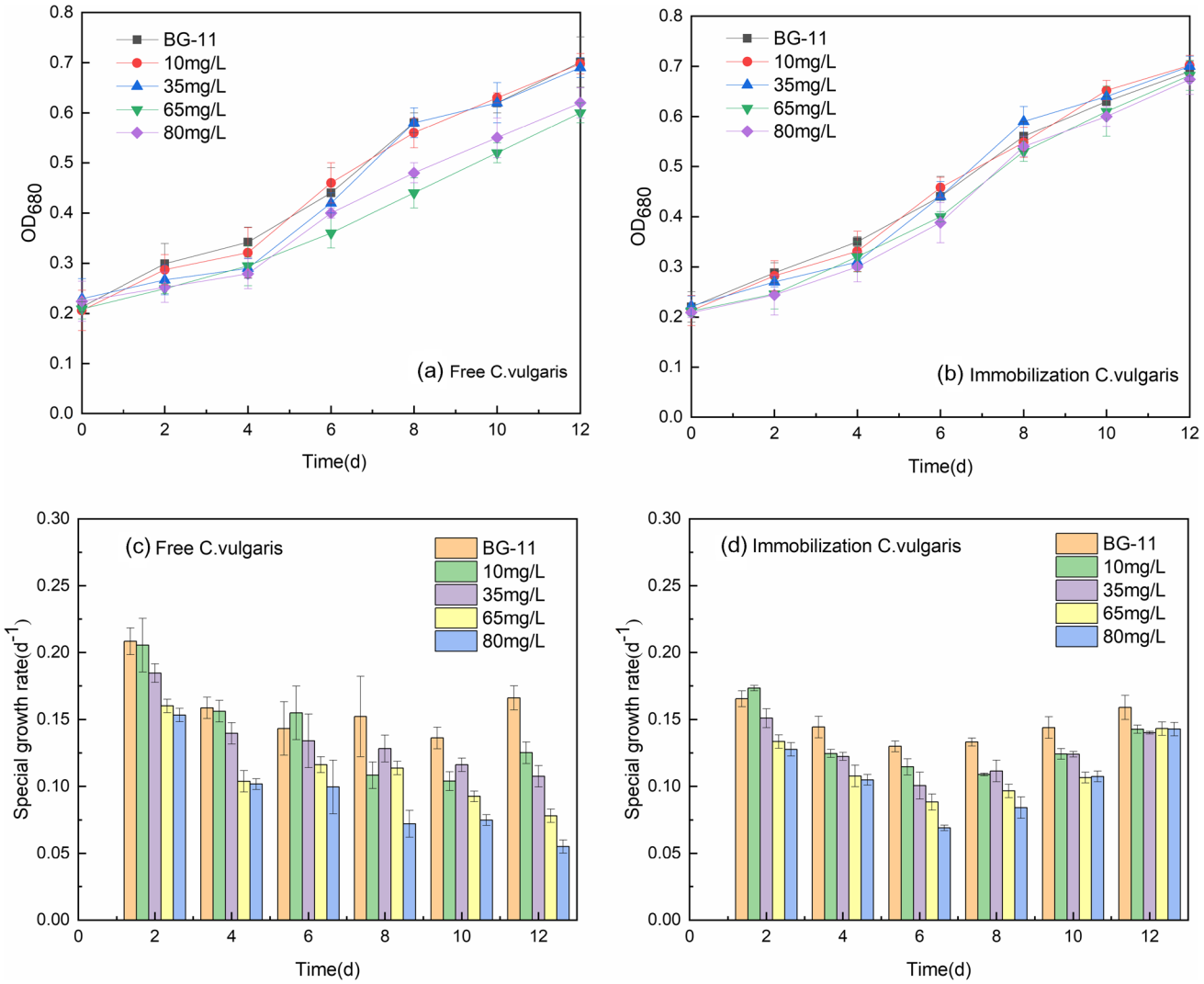

Fig. 1 Biomass variation and growth rate of free (**a**, **c**) and immobilized (**b**, **d**) *C. vulgaris* in the control and CBZ exposure groups

technology played a protective role in the energy conversion of microalgae growth. In conclusion, immobilization effectively alleviates the growth inhibition state of free microalgae and makes it possible to apply microalgae technology, especially under high carbamazepine exposure.

Photosynthetic activity variation

Photosynthesis is an essential process for microalgal growth and metabolism (Wang et al. 2020c). Microalgae absorb external inorganic carbon and light energy into cells through photosynthesis and then generate energy and organic carbon for the growth of microalgae. Chlorophyll is an important photosynthetic pigment for the conversion of light to chemical energy, allowing for light capture and energy transfer (Jin et al. 2019). In the initial experimental period (0–4 days), the chlorophyll contents of both free and immobilized microalgae at low CBZ concentrations (<50 mg/L) were similar to the control group, while on the 4th day, the chlorophyll contents at 10 mg/L of CBZ were significantly higher than those in the control group ($p<0.05$) (Fig. 2a, b). This indicates that the addition of low doses of CBZ could stimulate *C. vulgaris* growth and increase the chlorophyll content accordingly, a phenomenon referred to the hormesis effect (Liu et al. 2020). The promoting effect of chlorophyll content at low CBZ concentrations was mainly due to the antioxidation enzyme system response (Zhang et al. 2018), which successfully eliminated the reactive oxygen radicals accumulated in chlorophyll and thus increased the chlorophyll content of microalgae. During the cultivation period, the chlorophyll contents of immobilized *C. vulgaris* at different CBZ concentrations (0 mg/L, 10 mg/L, 35 mg/L, 65 mg/L, and 85mg/L) peaked on the 10th day, with concentrations of

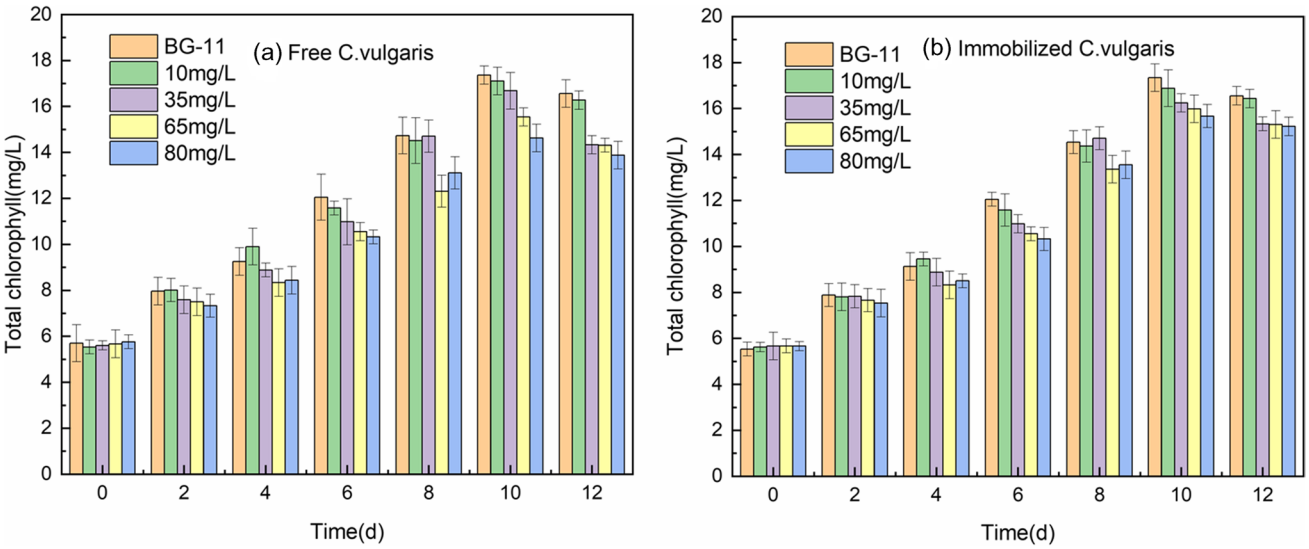

Fig. 2 Total chlorophyll variation in free (**a**) and immobilized (**b**) *C. vulgaris* exposed to varying concentrations of CBZ

17.36 mg/L, 17.12 mg/L, 16.26 mg/L, 15.52 mg/L, and 15.21 mg/L, respectively. And the chlorophyll contents of free microalgae also peaked on the 10th day, with the corresponding concentrations of 17.11 mg/L, 16.88 mg/L, 16.20 mg/L, 14.88 mg/L, and 14.32 mg/L. The highest chlorophyll concentration occurs on the 10th day because photosynthetic pigment is an important medium for the light-dependent reaction of microalgae (Almomani 2019), and the content of photosynthetic pigment decreases when the light energy utilization reaches saturation. The chlorophyll content exposed to low CBZ concentrations (<50 mg) was significantly higher than the group at high CBZ concentrations, and the gap continuously expanded (p<0.05). This phenomenon indicates that high CBZ concentrations affect the chlorophyll content of *C. vulgaris* and inhibit photosynthetic ability. The reduction in *C. vulgaris* chlorophyll content at high CBZ concentrations (>50 mg) may be caused by the degradation of PSII complexes and the peroxidation of thylakoid lipids (Li et al. 2019). As shown in Fig. 2 b, the inhibition of chlorophyll content by CBZ was significantly alleviated by immobilization with increasing cultivated time, especially at day 10 and day 12. This phenomenon may have resulted from the increased cell density after immobilization (Zhuang et al. 2020). The cluster effect caused by the denser concentration was beneficial to the substance exchange between microalgae cells and the obstruction of pollutants, thus contributing to the protection of the chlorophyll content and biomass growth. The higher chlorophyll content of immobilized microalgae compared with free microalgae also helps the microalgae stabilize the process of photosynthesis, release more dissolved oxygen into the environment, and improve the self-purification capacity of water (Almomani 2020a, 2020b; Chan et al. 2022).

Chlorophyll fluorescence determination is a noninvasive method for the evaluation of variation in photosynthetic activity, particularly when assessing the electron transfer rate in photosynthetic organisms subjected to environmental stress (Li et al. 2018; Wang et al. 2020b). Chlorophyll fluorescence analysis monitors various parameters, among which F_0 and F_M are highly sensitive to environmental pressure, with their measured values and derived data allowing analysis of PSII process activity. The parameter F_V/F_M represents the maximum potential quantum efficiency and the maximum light energy conversion efficiency of the PSII reaction center (Li et al. 2019). As shown in Fig. 3 a and b, exposure to low CBZ concentrations (from 10 to 35 mg/L) resulted in a slight decline between the control group and CBZ exposure group in both free and immobilized *C. vulgaris*. This finding is consistent with the variation in chlorophyll content, which indicated that exposure to a low concentration of CBZ did not significantly affect the photosynthetic activity of microalgae. In the initial exposure period (0–6 days), compared with the control group, the F_V/F_M of both free and immobilized microalgae decreased with the increase of time and the CBZ concentrations. At a concentration of 80 mg/L, the lowest values of F_V/F_M in the free and immobilized state were 55% and 76% of the control group, respectively. This phenomenon was due to the environmental stress caused by CBZ on microalgae cells, which inhibited their photosynthetic capacity. From the 6th to the 8th day of the experiment, the F_V/F_M value gradually increased with time, but there was a significant difference between the increasing amplitude of the immobilized and free states (p<0.05),

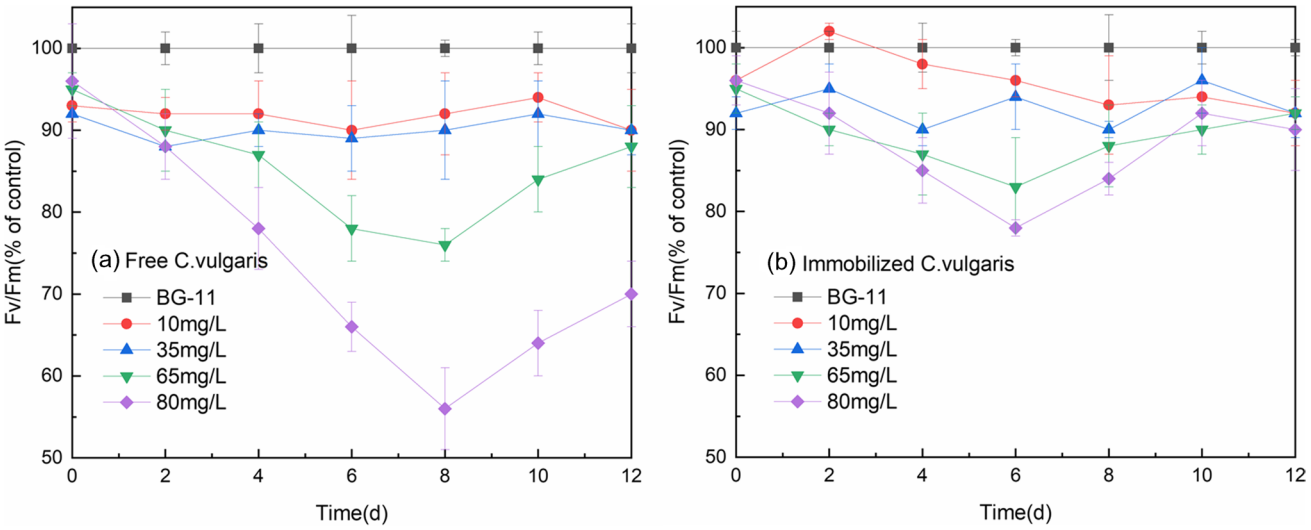

Fig. 3 Effect of varying concentrations of CBZ on the maximum quantum yield of PSII photochemistry (F_V/F_M) in free (**a**) and immobilized (**b**) *C. vulgaris*

especially at the high concentration group. These observations indicated that high CBZ concentrations (>50 mg/L) inhibited electron transport in the *C. vulgaris* photosystem and disrupted the protective mechanism response due to environmental stress (Wang et al. 2020a). Compared with the control group, the maximum F_V/F_M inhibition was 32% for the free microalgae, while the maximum inhibition for the immobilized microalgae was only 10%. This indicates that immobilization is beneficial for the protection of photosynthetic efficiency in *C. vulgaris* exposed to PPCPs pollutants. This result may be due to the combination of the growth of photosynthetic pigments promoted by the cluster effect and the photosynthetic activity provided by the low exposure concentration of immobilization mechanism to repair the active center of PSII.

Antioxidant response variation

During the growth and metabolism of algae, reactive oxygen species (ROS) play a key role in the cellular response to exogenous pollutants. However, excessive cellular ROS production damage the microbial membrane system, and eventually induce cell damage (Wang et al. 2020b). Therefore, antioxidant enzymes in microalgae are activated to eliminate excess ROS, and maintain a dynamic balance in microalgal cells under environmental pollution stress (Zhong et al. 2021). SOD is a typical antioxidant enzyme that is widely used as a signaling molecule indicating cell stress due to its role in transferring O^{2-} to H_2O_2 and O_2 to avoid cell damage by excess ROS (Zandalinas and Mittler 2018). As shown in Fig. 4, the SOD content gradually increases along with the increase of CBZ concentrations. The maximum content of SOD enzyme under free and immobilized *C. vulgaris* were 63 $U{\cdot}g^{-1}$ and 55 $U{\cdot}g^{-1}$, respectively. This phenomenon illustrated that the presence of CBZ stimulates the production of O^{2-} in *C. vulgaris* cells, leading to the activation of antioxidant enzymes for the protection of cells (Wang et al. 2020b). With the increase in CBZ concentration, excessive O^{2-} accumulation will be produced in microalgae, leading to the continuous improvement of SOD concentration. As shown in Fig. 4 a and b, immobilization did not affect the antioxidant enzyme system response, and both free and immobilized cells exhibited a similar response. This indicated that the antioxidant response was successfully activated in response to the toxic threat posed by carbamazepine.

MDA is the final product of membrane lipid peroxidation and was widely used to characterize and evaluate microalgal cell damage (Gonçalves et al. 2017). As shown in Fig. 4 a, the MDA contents of free *C. vulgaris* at 10 mg/L, 35 mg/L, 65 mg/L, and 80 mg/L of CBZ were 8 μmol/g,12 μmol/g, 22 μmol/g, and 28 μmol/g, respectively. MDA contents of free *C. vulgaris* exposed to high concentrations (65 mg/L and 80 mg/L) were significantly higher than the low concentrations (10 mg/L and 35 mg/L) ($p<0.05$). This indicated that the biochemical enzymes (such as SOD enzyme) produced by the antioxidative response of microalgae were not enough to completely eliminate excessive ROS at high CBZ concentrations, resulting in structural and functional damage. As shown in Fig. 4 b, the MDA contents of immobilized *C. vulgaris* at 10 mg/L, 35 mg/L, 65 mg/L, and 80 mg/L of CBZ were 7 μmol/g, 8 μmol/g, 11 μmol/g, and 13 μmol/g, respectively. The increased amplitude was not significant when compared to the control group. In comparison, the MDA content in free *C. vulgaris* increased significantly with increasing CBZ concentration, the maximum was up to 20 μmol/g. This phenomenon indicated that the immobilization

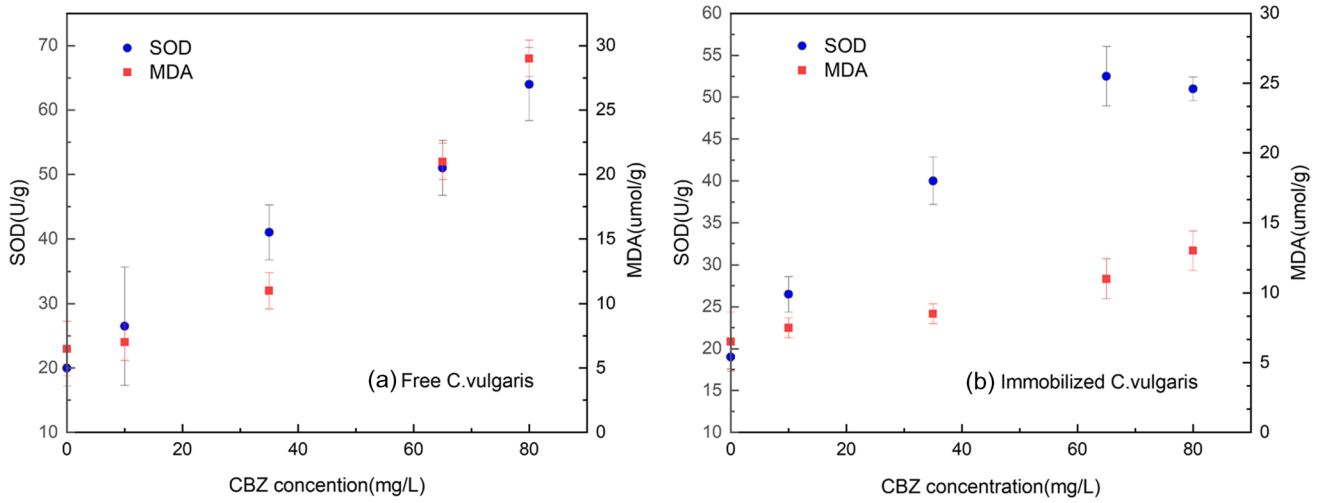

Fig. 4 Variation in MDA and SOD in free (**a**) and immobilized (**b**) *C. vulgaris* exposed to varying concentrations of CBZ

technique avoids the direct exposure of microalgae to CBZ and extends the transport time of pollutants through the substrate used for immobilization, thus forming an external condition outside the microalgae cells that is lower than the environmental concentration, allowing the antioxidant enzyme system to effectively reduce cell damage and protect *C. vulgaris* cells.

Effect of immobilization on CBZ removal

The CBZ removal rate by free *C. vulgaris* (FACHB-8) is illustrated in Fig. 5 a and 6 a. In the control group, no significant CBZ removal was observed, indicating that the removal of CBZ by abiotic processes was negligible, which is consistent with the findings of previous studies (Vo et al. 2019b). Considering the changes in SOD enzymes of microalgae, ROS in microalgae were altered and may be released into the environment. However, it was suggested that ROS had little contribution on PhCs removal by microalgae (Leng et al. 2020; Zhu et al. 2020). Therefore, the effects of ROS on CBZ removal were not considered in this study. After the 12th day of cultivation, the CBZ removal rate reached 90% at an initial CBZ concentration of 10 mg/L, while only 67% removal occurred at an initial CBZ concentration of 80 mg/L. At high concentrations, the environmental pressure of CBZ on microalgae inhibited its growth, and decreased photosynthetic pigment synthesis ability and physiological activity, which resulted in the inhibition of

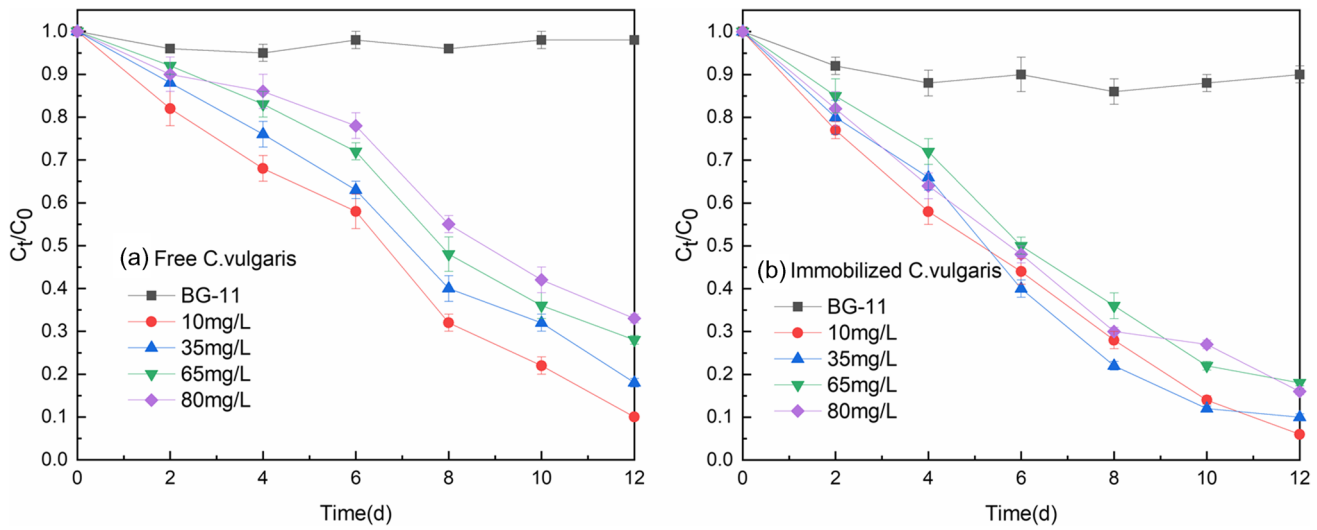

Fig. 5 Total removal rate (%) of CBZ by **a** free and **b** immobilized *C. vulgaris*

CBZ removal. In comparison, exposure to low CBZ concentrations allows *C. vulgaris* to effectively eliminate toxicity effects via cell metabolism and oxidative stress mechanisms. Thus, the growth of microalgae and the removal of CBZ are ensured. Organic pollutants are processed by microalgae via three main methods: biodegradation, bioaccumulation, and biosorption (Chan et al. 2022; Papazi et al. 2019). By measuring the CBZ concentrations on the outer surface of microalgal cells and within microalgal cells, the level of CBZ removal by biodegradation, biosorption, and bioaccumulation can be obtained (Song et al. 2019). The results show that biodegradation metabolism accounts for a significant level of CBZ removal in all CBZ concentration groups, contributing to more than 80% of CBZ removal. Biosorption accounted for 7–8% of the removal rate at all CBZ concentrations, while bioaccumulation contributed only 2–3% of removal. This phenomenon occurs because bioaccumulation is an active metabolic process in the uptake of pollutants driven by energy (Al Ketife et al. 2020). CBZ exposure damages the energy transfer ability in microalgae and thus inhibits bioaccumulation. Biosorption is mainly extracellular, and the sorption process determines significantly according to the functional groups of different PhCs and the microalgal species (Almomani and Bhosale 2021). Therefore, the biosorption removal ratio does not fluctuate significantly with changes in CBZ concentration. Biodegradation is the dominant removal pathway that occurs via intracellular and extracellular processes. The extracellular process mainly depends on the mass transport capacity of extracellular polymeric substances (EPS), while intracellular biodegradation is dominated by a complex enzyme system, which is easily influenced by environmental pollutant stress (Wang et al. 2020b). Hence, with the increase in CBZ concentration, the viability of microalgae cells decreases, the enzyme systems in cells are inhibited, and the metabolic capacity of the organisms is damaged. Meanwhile, cell damage at high concentrations will affect the release of extracellular polymer components, change their components, and slow down the transport efficiency of substances, thus reducing the removal efficiency of biodegradation metabolism.

The CBZ removal rate by immobilization is shown in Figs. 5 b and 6 b. In this study, microalgae are immobilized in PVA-SA beads, which can adsorb pollutants via an abiotic removal effect. The abiotic removal rate is found to be approximately 10% of the total CBZ concentration. Abiotic adsorption is mainly determined by the material structure and physicochemical properties of the beads, resulting in the adsorption amount remaining constant at different CBZ concentrations, as shown in Fig. 6 b. The removal of CBZ after immobilization treatment showed a trend of gradual increase over time, although with the increase in carbamazepine concentration, the final removal rate decreased from 95% in the 10 mg/L group to 87% in the 80 mg/L group. Compared to *C. vulgaris* in the free state, the removal efficiency and the removal proportion of the biodegradation pathway of immobilized cells were improved, especially in the high concentration groups, such as the 80 mg/L group, in which CBZ removal increased from 67 to 84%. The degradation of CBZ can be explained using a first-order kinetic model. As shown in Table 1, the degradation rate constants (k_1) of CBZ removal under free and immobilization states were comparable that ranged from 0.022 to 0.030 day^{-1}. k_1 in low concentration (< 50mg/L) was significantly higher than those in high concentration (> 50mg/L) ($p < 0.05$). The removal equilibrium capacity increased significantly under immobilization for each concentration ($p < 0.05$). The average increasement is about 25%. The removal efficiency improvement is due to the enhanced protective effects of microalgae by

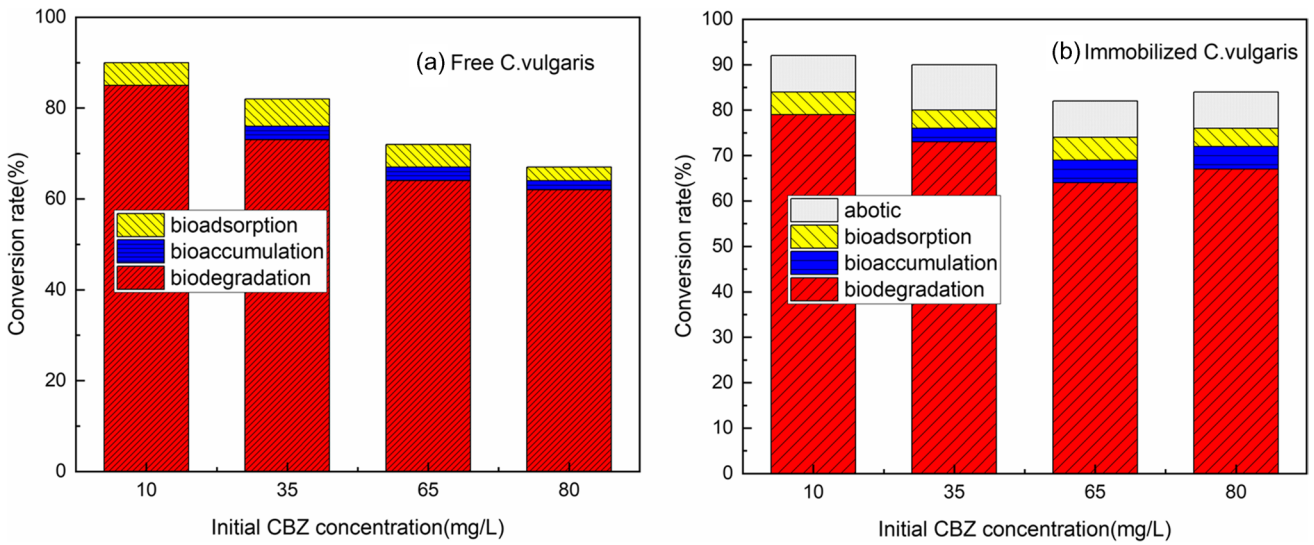

Fig. 6 Carbamazepine migration and distribution in free (**a**) and immobilized (**b**) *C. vulgaris* systems

Table 1 Parameters from fitting of the removal rate data with pseudo first-order kinetic model

	Free state microalgae			Immobilized microalgae		
	k_1 (day^{-1})	q_e (mg/g)	R^2	k_1 (day^{-1})	q_e (mg/g)	R^2
10mg/L	0.026±0.013	14.351±1.223	0.88	0.042±0.021	19.882±0.864	0.95
35mg/L	0.028±0.011	42.173±0.384	0.96	0.030±0.015	52.184±1.234	0.96
65mg/L	0.022±0.006	81.455±1.686	0.95	0.025±0.004	102.732±4.689	0.98
80mg/L	0.024±0.005	119.532±3.222	0.98	0.022±0.011	134.557±2.125	0.92

immobilization technology. The synergistic interaction between immobilized substrate and microalgae plays an important role in promoting *C. vulgaris* cell viability and biodegradation metabolic pathway stability. The immobilization of *C. vulgaris* in PVA-alginate beads allows cells to grow as an aggregation, resulting in a denser microalgal biomass in immobilization beads than free microalgae cultivation (Gojkovic et al. 2019). The denser microalgal content is beneficial to the bioaccumulation and biodegradation of CBZ. As shown in Fig. 6 b, biodegradation plays an important role and accounts for approximately 80% of CBZ removal. The improvement of removal efficiency at high concentrations indicates that immobilization can prevent microalgae from being directly exposed to high concentrations of CBZ and form an exposure environment with decreasing concentrations by immobilization substrate to protect the growth state and the antioxidative stress system of microalgae (Zhuang et al. 2020). In this way, the stability of intracellular transporter enzymes and metabolic capacity can be constructed, and the formation of stable substance transport channels between PVA-SA polymer and extracellular polymers can be promoted. The positive response of the antioxidant system can be helpful to avoid the excessive accumulation of ROS to damage the photosynthetic capacity and cell activity, thus facilitating the CBZ biodegradation. The less damaged microalgae under immobilization can effectively remove CBZ even under high concentrations.

Conclusion

The oxidative damage of *C. vulgaris* and their removal on CBZ in free and immobilized state were compared to explore whether immobilization can enhance the protective effects of microalgae and CBZ removal in this study. Results indicated that immobilization technology effectively protected the growth state of microalgae, alleviated the lipid peroxidation level, and promoted the repair of photosynthetic capacity, especially at high CBZ concentrations. After immobilization treatment, the CBZ removal rate was capable of reaching more than 90% at low CBZ concentrations and increased from 67 to 84% at 80 mg·L^{-1} CBZ concentrations. Both in immobilized and in free states, biodegradation was the dominant metabolic pathway of CBZ removal, accounting for more than 80% of the total CBZ removal rate. The cluster effect and interaction between immobilized substrate and microalgae were responsible for the improvement of microalgae protection and carbamazepine removal by immobilization technology. Our study provides a reliable pathway for removing degradation-resistant organic pollutant wastewater, especially at high concentrations. Further studies are needed to explore the protective effects and pollutant removal mechanisms of different environmental factors (such as pH, CO_2 concentration, and light intensity) on immobilized microalgae, so as to further contribute to the development of microalgae technology.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Zulin Hua, Xue Bai, and Lu Liang. The first draft of the manuscript was written by Lu Liang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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